

**HEAT SHOCK PROTEINS: TREATING METABOLIC DISEASE BY TURNING UP
THE HEAT**

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ABSTRACT

The prevalence of type 2 diabetes has risen as access to nutrition as increased and physical activity levels have decreased. Low cardiorespiratory fitness (CRF) levels are influenced by genetics, but low levels of physical activity also drive down levels of CRF in individuals increasing their susceptibility to metabolic disease. Heat shock proteins (HSP) are anti-inflammatory, anti-apoptotic molecular chaperones that assist in refolding proteins that have become unfolded during stress. HSP72 expression is lower in multiple tissues of obese, type 2 diabetics and HSP72 ablation results in insulin resistance.

The work here confirms that *in vivo* heat treatments and the induction of HSP72 improve whole-body glucose homeostasis, skeletal muscle insulin sensitivity, and suggest that heat treatment may protect against metabolic derangements in the white adipose tissue and liver. In the WAT, constitutive HSP levels and the induction of HSPs following heat treatment is different between WAT depots with abdominal WAT showing greater constitutive HSP levels but a lower induction of HSPs following heat treatment. Heat treatment also modified fatty acid handling in the WAT in a depot specific fashion.

Using a rat model to mimic intrinsic low CRF observed in diabetic humans, we examine the metabolism of rats selectively bred to be low capacity runners (LCR) or high capacity runners (HCR). We observed that LCR rats are particularly susceptible to skeletal muscle insulin resistance following an acute (3 d) high-fat diet challenge, particularly in the glycolytic muscle where we observed lower HSF-1, HSP72, and mitochondrial proteins. In the liver and skeletal muscle, a 3 d HFD reduced the induction of HSPs following heat treatment. Finally, we found evidence that supports a role for HSP72 in maintaining proper autophagy-mitophagy that may

result in an improved pool of functional mitochondria. This work strongly suggests that that HSP72 plays a role in protection against insulin resistance and metabolic derangements induced by high-fat feeding in multiple organs intimately tied with whole-body glucose homeostasis.

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IN MEMORY OF

Ashlee Nicklow Rogers, Nov 6 1982 – Oct 20 2012. "Forever in our hearts"

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ABBREVIATIONS

ACC, acetyl CoA carboxylase
ADP, adenosine diphosphate
AMP, adenosine monophosphate
AMPK, adenosine monophosphate activated protein kinase
ANOVA, analysis of variance
AS160, Akt substrate of 160 kDa (also known as TBC1D4)
ATGL, adipose triglyceride lipase
BAT, brown adipose tissue
BMI, body mass index
CaMKII, calcium/calmodulin-dependent protein kinase II
CDC, Center for Disease Control & Prevention
CPT-1, carnitine palmitoyltransferase-1
CRF, cardiorespiratory fitness
CRP, C-reactive protein
CSF-1, monocyte colony stimulating factor-1
CVD, cardiovascular disease
DAG, diacylglycerol
DNA, deoxyribonucleic acid
ER, endoplasmic reticulum
ETC, electron transport chain
eWAT, epididymal white adipose tissue
FADH₂, flavin adenine dinucleotide
FAS, fatty acid synthase
FFA, free fatty acids
GAP, GTP-activating protein
GGA, geranylgeranylacetone
GLUT2, glucose transporter 2
GLUT4, glucose transporter 4
GSK3, glycogen synthase kinase-3
GDP, guanosine diphosphate
GTP, guanosine triphosphate
GWAS, genome wide association study
H₂O₂, hydrogen peroxide
HbA1c, hemoglobin A1C
HCR, high capacity runners (HCR rats)
HDL, high density lipoprotein ('Good' cholesterol)
HSE, heat shock response elements
HSL, hormone-sensitive lipase
HRP, horseradish peroxidase
IKK, inhibitor of kappa B kinase
IL-1 β , interleukin-1 β
IL-6, interleukin-6
IP₃, inositol 1,4,5-triphosphate
IRS-1/2, insulin receptor substrate-1/2

JNK, c-Jun N-terminal kinase
 LC3, microtubule-associated protein 1 light chain 3
 LCR, low capacity runners (LCR rats)
 LKB1, liver kinase B1
 MAPK, mitogen-activated protein kinase
 MCP-1, monocyte chemotactic protein-1
 MET, metabolic equivalent (1 MET = resting)
 mtDNA, mitochondrial deoxyribonucleic acid
 mTORC1, mammalian target of rapamycin complex 1
 mTORC2, mammalian target of rapamycin complex 2.
 NADH, nicotinamide adenine dinucleotide
 NASH, non-alcoholic steatohepatitis
 NCEP, National Cholesterol Education Program
 NEFA, non-esterified fatty acids
 NF- κ B, nuclear factor kappa B
 NHANES, National Health and Nutrition Survey
 NIH, National Institutes of Health
 PAI-1, plasminogen activator inhibitor-1
 RBP-4, retinol binding protein-4
 PDE3B, phosphodiesterase-3B
 PDK1, phosphatidylinositol-dependent kinase 1
 PDK4, pyruvate dehydrogenase kinase-4
 PE, phosphatidylethanolamine
 PEPCK, phosphoenolpyruvate carboxylase
 PGC-1 α , peroxisome proliferator-activated receptor γ coactivator 1 α
 PGC-1 β , peroxisome proliferator-activated receptor γ coactivator 1 β
 PH, pleckstrin homology
 PI3K, phosphatidylinositol 3-kinase
 PIP₂, phosphatidylinositol-3,4 bisphosphate
 PIP₃, phosphatidylinositol-3,4,5 triphosphate
 PKA, protein kinase A
 PKB, protein kinase B (also known as Akt)
 PKC, protein kinase C
 PPAR γ , peroxisome proliferator-activated receptor γ
 PPRC1, peroxisome proliferator-activated receptor γ coactivator-related 1
 PTB, protein tyrosine-binding
 PTEN, phosphatase and tensin homolog
 RBP-4, retinol binding protein-4
 RER, respiratory exchange ratio
 rpWAT, retroperitoneal white adipose tissue
 ROS, reactive oxygen species
 SCAT, subcutaneous white adipose tissue
 SH2, Src-homology-2
 Sirt1, sirtuin-1
 SR, sarcoplasmic reticulum
 TAK-1, transforming growth factor β activated kinase-1

TCA cycle, tricarboxylic acid cycle

TNF α , tumor necrosis factor α

TRPV4, transient receptor potential cation channel subfamily V member 4

UCP-1, uncoupling protein-1

ULK1, Unc-51 like autophagy activating kinase 1

WAT, white adipose tissue

WHO, World Health Organization

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CHPATER 1

INTRODUCTION

1.1. DIABETES & INSULIN RESISTANCE

Diabetes mellitus is a metabolic disorder characterized by the loss of insulin secretion (insulin deficiency – type 1 diabetes) or loss of insulin action (insulin resistance – type 2 diabetes). The resulting disruptions lead to hyperglycemia damaging organs ultimately leading to premature death. Type 1 diabetes is characterized by the autoimmune destruction of insulin secreting β -cells of the pancreas resulting in a loss of circulating insulin and hyperglycemia (42, 249). Type 1 diabetes is often diagnosed in childhood at which time lifelong insulin treatment is initiated to maintain euglycemia (42). Type 1 diabetes makes up 5%-10% of all cases of diabetes and had previously been referred to as “insulin-dependent diabetes mellitus” or “juvenile diabetes.”

The majority of diabetic cases (90%-95%) are type 2 diabetes. Type 2 diabetes is characterized by the loss of insulin action resulting in hyperinsulinemia, post-prandial glucose intolerance, and moderate hyperglycemia depending on the severity. This form of diabetes was formerly referred to as “non-insulin-dependent diabetes mellitus” or adult onset diabetes mellitus.” The loss of insulin action is often referred to as insulin resistance as insulin’s intracellular signal transduction is impeded by a variety of mechanisms. Insulin resistance in the peripheral tissues results in compensation by the pancreatic β -cells to produce more insulin in order to maintain euglycemia. The skeletal muscle is responsible for approximately 75%-80% of post-prandial glucose disposal (39, 115), and insulin resistance in the skeletal muscle is an early step that may trigger disruption of glucose homeostasis (475). Diabetes is the seventh leading cause of death in the U.S., although rates may be underreported as diabetes status is under recorded on death certificates (58, 310). Diabetes (both type 1 and 2) is associated with increased risk of death and development of a host of other diseases including heart disease, stroke,

blindness and other retinopathies, kidney disease, neuropathies, neurodegeneration, amputations, and non-alcoholic fatty liver disease (NAFLD), amongst others (145, 146, 472, 542).

1.1.1. Epidemiology & Impact. In the last 20 years, the prevalence of type 2 diabetes has risen steadily as access to excess nutrition has grown with concurrent wide spread low levels of physical activity. The global prevalence of type 2 diabetes has doubled since 1980 to approximately 357 million in 2008 (87), with the largest rises occurring in the Middle Eastern Crescent, sub-Saharan Africa, and India (366). Thus, type 2 diabetes has become a global concern. In the U.S., the prevalence of type 2 diabetes has risen sharply in the last 50 years from approximately 1% in 1958 to 9.3% (29.1 million) in 2012 (58). Another 86 million U.S. adults over the age of 20 years are pre-diabetic, defined as fasting blood glucose concentration between 100-125 mg/dl, hemoglobin A1c (HbA1c) 5.7%-6.4%, or impaired glucose tolerance, putting these individuals at elevated risk of developing type 2 diabetes (58). Alarming, the U.S. Center for Disease Control and Prevention (CDC) has predicted that one in three children born after 2000 will develop type 2 diabetes during their lifetime (76). As the prevalence of diabetes is expected to rise in the U.S. and throughout the world, novel prevention strategies and treatments are desperately needed to slow the prevalence and improve quality of life for patients.

1.1.2. Etiology. Type 2 diabetes results from the progressive loss of insulin action over many years due to peripheral insulin resistance leading to pancreatic β -cell dysfunction and apoptosis culminating in the loss of insulin production. Early in the disease process, the loss of insulin sensitivity in the primary metabolic tissues (i.e., skeletal muscle, adipose tissue, and liver) results in impaired post-prandial glucose clearance and compensatory hyperinsulinemia to maintain euglycemia. Loss of skeletal muscle insulin sensitivity results from defects in

intracellular insulin signal transduction (85), defects in glucose transporter content and action (176), and ‘lipotoxicity’ (409). In addition to derangements at the skeletal muscle level, hepatic insulin resistance results in a loss of insulin’s ability to augment hepatic glucose production following a meal while resulting hyperinsulinemia stimulates hepatic *de novo* lipogenesis (428).

As whole-body insulin resistance progresses, there is a loss of insulin secretion by pancreatic β -cells resulting from deposition of amyloid protein in pancreatic islets (203), oxidative stress (413), lipotoxicity (73), and a lack of incretin effects (7, 8). The progression from impaired insulin sensitivity to the loss of insulin production takes many years (i.e., 10-25 years) with a large amount of individual differences in the time to reach pancreatic β -cell failure with approximately 50% of individuals requiring insulin treatment within 10 years (496). It is important to note going forward, that lifestyle interventions that include increased levels of exercise and physical activity, as well as 5%-10% reductions in body weight are effective at alleviating diabetic symptomology (21, 250, 366, 456).

1.1.3. Risk Factors. The risk of developing type 2 diabetes is multifactorial and a number of risk factors have been identified including obesity, ethnicity, socio-economic factors, family history, genetics, epigenetics, a history of gestational diabetes, high/low birth weight, age, physical inactivity, and low cardiorespiratory fitness (CRF) (34, 104, 171, 196, 253, 258, 453, 514).

1.1.3.1. Ethnicity. In the U.S., minorities are at elevated risk for developing pre-diabetes and type 2 diabetes. The prevalence of diabetes is 7.6% in non-Hispanic white adults, 9.0% in Asian Americans, 12.8% in Hispanics, 13.2% in African Americans, and 15.9% in Native American Indians and Alaskans (58). Many individuals within these ethnicities live in

relative poverty, thus socio-economic status cannot be ignored, as it is also a risk factor for developing type 2 diabetes. However, even within the same neighborhoods, Mexican Americans have a greater prevalence of type 2 diabetes than their Caucasian neighbors (156). This highlights that ethnicity itself is important in assessing an individual's risk for developing type 2 diabetes.

1.1.3.2. Genetics. There is no doubt that genetics plays a role in the risk of developing type 2 diabetes. During the 1960s, James V. Neel hypothesized that a thrifty gene would be discovered that explains ethnic differences in susceptibility to obesity and type 2 diabetes (349). This hypothesis has been revised to a 'thrifty phenotype' as it became clear that multiple genes would interact with the environment leading to disease. Heritability studies have shown that the concordance rate of type 2 diabetes is approximately 70% between monozygotic twins and 20%-30% in dizygotic twins (393). Furthermore, the risk of diabetes if one parent has type 2 diabetes is 3-fold higher compared to if no parents are affected, but 6-fold higher if both parents are affected (315). These studies highlight that inherited risk and genetics certainly play a role but cannot alone explain a large portion of the risk.

In the last 10 years, the combination of multiple genome-wide association studies (GWAS) has described over 60 genetic loci associated with type 2 diabetes (34, 393). From these studies, a number of candidate genes have been shown to be associated with the risk of developing type 2 diabetes including *PPARG*, *KCJN11*, *TCF2*, *TCF7L2*, *CDKAL1*, *CDKN2A-CDKN2B*, *IGFBP2*, to name a few (393). The odds ratio of each independent loci is between 1.10-1.20 or less. Therefore, the risk due to an individual gene loci is relatively small. In addition, of the loci identified, inheritability explains less than 20% (393). Thus, the interaction

of an individual's genome with environmental factors is likely to play the largest role in the development of type 2 diabetes making environmental and lifestyle choices paramount.

1.1.3.3. Aging. Insulin resistance is known to increase with advancing age as insulin sensitivity falls throughout an individual's life (93). Although the exact mechanisms are not known, aging is associated with increased production of reactive oxygen species (ROS), decreased antioxidant protection, lower heat shock protein (HSP) levels (157, 158, 441), mitochondrial dysfunction, reduced mtDNA levels, and increased damage to mtDNA (25, 319, 446, 455, 518). Reduced mitochondrial function has been implicated in the development of insulin resistance and diabetes and will be discussed below (376). The loss of insulin action during aging has also been intimately associated with neurodegenerative diseases including Alzheimer's disease.

1.1.3.4. Obesity. Currently two-thirds of the U.S. adult population are overweight and one-third are obese assessed by body mass index (BMI, calculated as weight divided by height squared) (119). The CDC and WHO identify a BMI between 25.0 and 29.9 kg/m² in adults as overweight and ≥ 30 kg/m² as obese. Two alarming statistics should be noted; first, the number of individuals classified as morbidly obese (BMI > 40 kg/m²) is rapidly rising and has now reached a prevalence of 3% of the U.S. adult population (463, 464). Second, 16.9% of all children and adolescents aged 2-19 years are considered obese (BMI \geq 95th percentile of the BMI-for-age growth charts) (360). It has become recognized that increased amounts of adipose tissue located in the abdominal cavity around the internal organs, known as visceral adipose tissue, confers greater risk of developing insulin resistance and type 2 diabetes compared with levels of subcutaneous adipose tissue (121, 159).

1.1.3.5. Sedentary lifestyle. Generally it is recommended that adults accumulate a minimum of 150 min/wk of moderate intensity exercise and accumulate 30 min/d of physical activity. However, in modern society many individual engage in variety of sedentary behaviors in addition to not meeting these recommendations. Sedentary behaviors include not participating in recommended amounts of weekly exercise, a lack of daily physical activity, as well as extended periods of inactivity (i.e., sitting at a desk, television watching). A number of reports have linked physical inactivity, sedentary behaviors, and sitting time to chronic diseases including hypertension (198, 365, 381), obesity (221, 272, 491, 492), type 2 diabetes (51, 171, 195, 267), metabolic syndrome (103, 212), CVD, and all-cause mortality (38, 197, 199, 200, 284, 364, 444, 454).

In the last 50 years, leisure-time physical activity has not changed, but occupational-related physical activity has decreased sharply (72). As a result, U.S. adults spend 7.7 hr of their waking day sitting or lying (305). Bouts of activity interspersed with long periods of sitting are commonplace, and recent evidence suggests that long bouts of sitting have negative health consequences independent of physical activity levels (103, 160, 196) and participation in moderate-to-vigorous intensity exercise (259). Thus, unfortunately, even those individuals meeting the recommended exercise goals, but spending large portions of their day sedentary, still have elevated risk (499).

A number of trials have demonstrated that exercise combined with diet-induced weight loss is a powerful tool in preventing the development of type 2 diabetes (250, 367, 494). Exercise or increased physical activity can reduce hyperglycemia, improve glucose tolerance, and reduce HbA1c levels in type 2 diabetic patients (19, 21, 22, 68, 231). Similarly, breaking up sitting time

can also improve glucose homeostasis (102). Thus, exercise, high levels of physical activity, and reduced periods of sitting can all improve aspects of diabetic symptomology and help prevent the development of type 2 diabetes (21, 456).

1.1.3.6. Cardiorespiratory fitness. Cardiorespiratory fitness (CRF) can loosely be described as the body's ability to take in oxygen, deliver it to the peripheral tissues, and the ability of the peripheral tissues to extract and utilize this oxygen. Individuals with a high level of CRF have lower risk of all-cause mortality (40, 41, 69), CVD mortality (40, 41, 71), hypertension (26, 398), obesity and abdominal obesity (416, 527), metabolic syndrome (270, 273, 447), and type 2 diabetes (57, 253, 298, 436, 448, 514). Kodama *et al.* reported in a meta-analysis of 33 studies that every 1 MET (metabolic equivalent) increase in CRF results in a 13%-15% reduction in all-cause and CVD mortality (253). The study of the mechanisms by which CRF employs this protection is difficult because of the number of metabolic pathways that are influenced in parallel.

CRF is influenced by many factors including training and physical activity levels, but genetics account for > 60% of the equation (46). An interesting model to study the relationship between CRF and susceptibility to metabolic diseases is using rats that have been selectively bred as high capacity runners (HCR) or low capacity runners (LCR). In brief, Steve Britton and Lauren Koch bred rats based on their endurance running capacity for successive generations to determine how intrinsic CRF levels influence disease susceptibility. The founder population consisted of rats from a genetically heterogeneous N:NIH rat stock which had been developed by breeding eight inbred rat strains together (162, 251, 252). Male and female rats from this founder population were then tested for endurance running capacity on treadmills. The top 13 (out of 100

tested) male runners were mated with the top 13 female runners, and vice versa for the low performing rats, to create 13 breeding families of HCR and LCR rats. This breeding scheme has been continued for successive generations. After initial testing to determine phenotype for breeding, the rats were returned to sedentary, inactive conditions. Thus, the initial testing was the only exercise stimulus the rats received.

Early the breeding it became clear that a disease phenotype had appeared in the LCR rats. The LCR rats showed symptomology of metabolic syndrome including hypertension, elevated fasting blood glucose and insulin concentration, dyslipidemia, post-prandial glucose intolerance, whole-body and skeletal muscle insulin resistance, and hepatic steatosis (285, 286, 332, 347, 355, 357, 407, 442, 476, 525), and these conditions are exacerbated in LCR rats by high-fat feeding while HCR rats are protected against diet-induced metabolic disruptions (132, 355, 357, 503). At the skeletal muscle level, LCR rats have lower insulin-stimulated glucose uptake and lower mitochondrial functionality and content (285, 286, 347, 355, 407, 442). As lower mitochondrial function and content have been implicated in insulin resistance and type 2 diabetes (376), the skeletal muscle of the LCR rats may be particularly susceptible to metabolic insults. Thus, there is no doubt that low CRF puts an individual at risk for developing metabolic diseases, and it is important to map out the mechanisms by which this susceptibility occurs to fully understand how high CRF protects against insulin resistance.

1.2. INSULIN & INSULIN SIGNALING

The discovery of insulin was truly a game changer in medicine for the treatment of diabetes. Before the discovery of insulin, diabetes was a wasting disease resulting in emaciation and early death. Insulin's major role is to regulate blood glucose levels in normal ranges [fasting glucose 4 mM – 7 mM, 80 – 100 mg/dl], and to control post-prandial glucose redistribution from the circulation into the peripheral tissues. Type 2 diabetes is characterized as fasting blood glucose > 126 mg/dl and pre-diabetes is defined as fasting blood glucose between 100-125 mg/dl.

1.2.1. History of diabetes & insulin. Descriptions of diabetic patients can be dated back to the ancient Egyptians (1500 BCE) (137, 392). The word 'diabetes' derives from the Greek word siphon as the Greek physician Aretaeus of Cappadocia described patients having polydipsia concurrent with polyuria (137). The word 'mellitus' is derived from the Latin for "honey sweet" because of the sweet tasting urine and blood in these patients (137). It was not until the late 19th century and early 20th century when insulin was discovered and determined to be the root cause of diabetes was described and treatment options become a reality.

Edward Albert Sharpey-Schafer hypothesized that the loss of a single chemical produced from the pancreas was responsible for diabetes, calling this chemical *insula*, meaning 'land' (392). Frederick Banting and Charles Best discovered insulin in the 1920s (24, 392), and along with James Collip and John Macleod, purified bovine insulin and successfully treated patients using insulin (138, 392). These accomplishments resulted in Banting & Macleod being awarded the 1923 Nobel Prize in Medicine. A description of type 2 diabetes emerged as Harold Himsworth in 1936 described that insulin resistance, not insulin deficiency, occurs in some

diabetic patients (179). Later in 1988, Gerald Reaven described a constellation of metabolic disorders that put an individual at increased risk for developing diabetes including insulin resistance, upper body obesity, hypertension, hypertriglyceridemia, and low high-density lipoprotein (HDL) levels (399, 400). These discoveries set the stage for the piecing together of the complicated intracellular insulin signaling pathway that would occur as it became accepted that insulin signal and that this signal transduction was blunted in insulin resistant type 2 diabetic patients.

1.2.2. Insulin release. Following a meal, insulin secretion from the pancreatic β -cells increases circulating levels of insulin. The release of insulin occurs in two phases. The first phase occurs rapidly in response to rising blood glucose levels following a meal. The second phase is more sustained arising from glucose-independent signals. Glucose entry into β -cells through glucose transporters, specifically glucose transporter-2 (GLUT2), increases ATP levels, closes ATP-sensitive potassium channels, increases intracellular potassium levels ultimately, resulting in depolarization of the plasma membrane. Depolarization results in opening of voltage-gated Ca^{2+} -channels leading to an influx of Ca^{2+} ions from the extracellular fluid. This influx of Ca^{2+} ions activates phospholipase C which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 -gated channels of the SR releasing greater amounts of Ca^{2+} into the cytosol. The increase in cytosolic Ca^{2+} concentration induces the translocation of insulin secretory vesicles to the plasma membrane releasing insulin into the circulation.

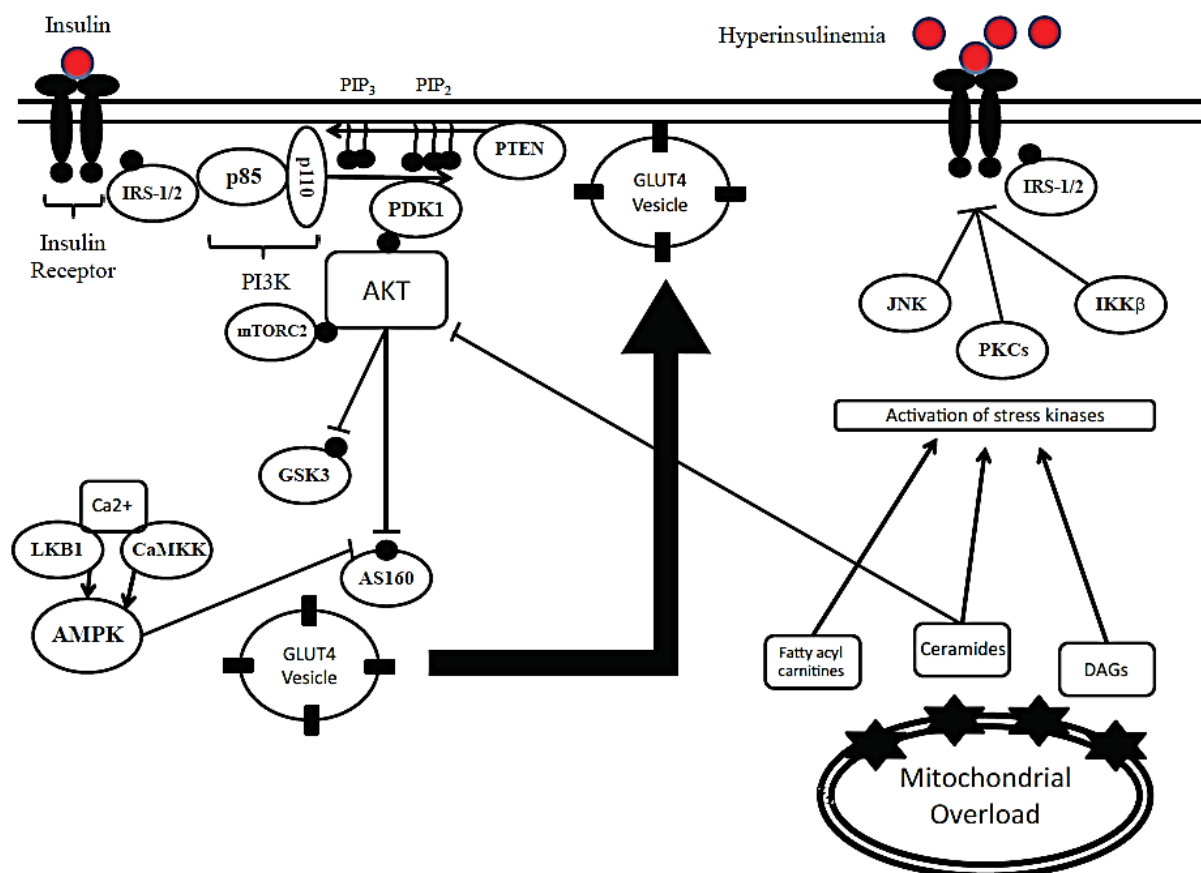


Figure 1. Insulin signaling pathway activation results in translocation of GLUT4 to the plasma membrane to facilitate glucose uptake in skeletal muscle and adipocytes.

Abbreviations: Akt (protein kinase B, PKB), AMPK (AMP-activated protein kinase), AS160 (Akt-substrate of 160 kDa), CaMKK β calcium/calmodulin-dependent protein kinase II), DAGs (diacylglycerols), GLUT4 (glucose transporter-4), GSK3 (glycogen synthase 3), IKK β (inhibitor of kappa B kinase subunit β), IRS-1/2 (insulin receptor substrate), LKB1 (liver kinase B1), JNK (c-Jun N-terminal kinase), mTORC2 (mammalian target of rapamycin complex 2), PDK1 (phosphatidylinositol-dependent kinase 1), PI3K (phosphatidylinositol 3-kinase), PIP₂ (phosphatidylinositol-3,4 bisphosphate), PIP₃ (phosphatidylinositol-3,4,5 triphosphate), PTEN (phosphatase and tensin homolog).

1.2.3. Insulin Signaling. Circulating insulin binds to its plasma membrane receptor resulting in activation of an intracellular signaling cascade that in adipocytes and myocytes results in increased glucose uptake from the circulation into these cells. More specifically, insulin induces the translocation of glucose transporter molecules to the plasma membrane from intracellular storage compartments (84, 469). This transporter was later identified as glucose transporter-4 (GLUT4) (36, 218, 219). Over the course of many years the molecules involved in transmitting insulin's signal from the plasma membrane to the intracellular compartments containing GLUT4 molecules have been mapped.

The plasma membrane insulin receptor itself is a tyrosine kinase composed of two extracellular α -subunits that bind insulin, and two transmembrane β -subunits with intrinsic tyrosine kinase activity (35, 74, 232, 389). Insulin binding to the α -subunits results in auto-phosphorylation of tyrosine residues on the β -subunits further activating the receptor's kinase activity (1, 27, 35). The insulin receptor then phosphorylates effector proteins such as insulin receptor substrate-1/2 (IRS-1/2) (417, 465, 522). The IRS proteins contain a pleckstrin homology (PH) domain, protein tyrosine-binding (PTB) domain, and numerous tyrosine residues (35). Upon insulin stimulation and IRS phosphorylation by the insulin receptor, IRS-1/2 associates with phosphatidylinositol 3-kinase (PI3K) increasing the activity of PI3K leading to the conversion of PIP_2 to phosphatidylinositol-3,4,5 triphosphate (PIP_3) (20, 418, 487, 488). PI3K consists of a regulatory p85 subunit and a catalytic p110 subunit, and it is the Src-homology-2 (SH2) domain of the p85 subunit that facilitates the interaction between IRS-1/2 and PI3K (74). The PI_3 -phosphates generated by PI3K target a number of downstream molecules including PI-dependent kinase 1 (PDK1), glycogen synthase kinase-3 (GSK3), protein kinase C (PKC),

proteins involved in actin remodeling, and protein kinase B (PKB)/Akt (35, 52, 74, 79, 122, 248, 520).

Akt is essential for transduction of insulin's signal intracellularly, has a diverse number of targets, and plays a central role in several intracellular signaling pathways (62, 74). Upon insulin stimulation, Akt is recruited to the plasma membrane and interacts with PIP₃ via its PH domain, but this interaction alone is not responsible for the activation of Akt itself (220). Rather, Akt is phosphorylated on Thr308 and Ser473 residues as a result of insulin receptor activation (10), with PDK1 responsible for phosphorylating Thr308 (11, 461) and mammalian target of rapamycin complex 2 (mTORC2) responsible for phosphorylating Ser473 (193, 433). With respect to glucose uptake, Akt phosphorylates the Rab GTPase activating protein (GAP) AS160 (also known as TBC1D4) which has been described as the brake to insulin signaling (248). AS160 is a GAP that promotes cognate Rab GTPases to hydrolyze GTP and remain inactive (248, 322, 431). Phosphorylation of AS160 by Akt eliminates AS160's GAP activity allowing Rab proteins to become active by exchanging GDP for GTP inducing GLUT4 translocation. AS160 mutants that cannot be phosphorylated by Akt or knockdown of AS160 *in vitro* results in a constitutively active AS160 that blocks GLUT4 translocation to the plasma membrane (61, 213, 431, 466).

Akt not only induces translocation of GLUT4 molecules to the plasma membrane, Akt activates glycogen synthesis by phosphorylating GSK3 α on Ser21 and GSK3 β on Ser9 resulting in inhibition of GSK3 activity (78, 311). Constitutively GSK3 α/β phosphorylates and inhibits glycogen synthase reducing glycogen synthesis. However, when Akt inhibits GSK, GSK3's inhibition over glycogen synthase is relieved allowing glycogen synthesis to become active.

Glucose uptake is also stimulated by other stressors including muscle contractions and increased intracellular Ca^{2+} levels. This represents a redundant biological pathway by which glucose uptake is stimulated independent of insulin. A number of molecules have been shown to activate insulin signaling independent of insulin including calcium/calmodulin-dependent protein kinase II (CaMKII), AMP-activated protein kinase (AMPK), protein kinase C (PKC) isoforms α , β , γ , and ζ , and p38 mitogen-activated protein kinase (MAPK) (136, 384, 528). AMPK has received a great deal of attention for its many metabolic roles. AMPK is an ancient energy sensor that senses increases in AMP and ADP levels within the cell and phosphorylates downstream targets ultimately resulting in increased ATP production to maintain energy homeostasis (163, 226). Activation of AMPK has wide spread biological effects including decreased glycogen synthesis and increased glycolysis (304), decreased fatty acid and cholesterol synthesis (55, 56, 130), decreased lipolysis (127, 130, 131), increased fatty acid oxidation (321, 501, 523, 524), and increased mitochondrial biogenesis (226, 430). Upon activation, AMPK targets downstream proteins including AS160, which AMPK phosphorylates and inhibits allowing for GLUT4 translocation to the plasma membrane.

1.2.4. Insulin resistance. The loss of insulin action resulting in type 2 diabetes begins long before diagnosis. The resistance to insulin affects multiple tissues, but in the context of the early stages in the development of type 2 diabetes, insulin resistance largely affects the skeletal muscle, liver, and adipose tissue. It has become clear that blunted insulin signal transduction is a key aspect of type 2 diabetes. A number of mechanisms are being actively studied as the cause of insulin resistance and blunted insulin signal transduction including, but certainly not limited to, inflammation and activation of intracellular pro-inflammatory pathways, generation of fatty acid metabolites, and mitochondrial abnormalities (408). Although the skeletal muscle is responsible

for most of post-prandial glucose disposal, evidence from animal models suggest that altered liver metabolism and reduced insulin sensitivity occur prior to skeletal muscle following high-fat feeding (262, 427). Thus, resistance to insulin in the skeletal muscle and liver has been widely studied although the adipose tissue's role is often underappreciated.

1.2.4.1. Inflammation. A state of low-grade, chronic inflammation has been postulated to occur early in the etiology of type 2 diabetes and possibly cause aspects of metabolic dysfunction (519). It had been known for many years that anti-inflammatory salicylates (e.g., aspirin) could restore insulin signaling (129, 536). This hypothesis was supported by the findings that a circulating inflammatory cytokine, tumor necrosis factor α (TNF α), secreted from immune cells and adipocytes could itself induce insulin resistance. TNF α levels are elevated in obese humans (192, 241, 422), and knockout of TNF α or its receptor results in protection against obesity (192, 497). Subsequently, TNF α was shown to activate intracellular pro-inflammatory stress kinases (i.e., PKC, c-Jun N-terminal kinase, JNK, and inhibitor of kappa B kinase β , IKK β) that phosphorylate inhibitory serine residues on IRS-1/2 blocking insulin signal transduction (5, 6, 90, 147, 191, 378, 536, 541). Several other circulating cytokines/adipokines have also been described to be pro-inflammatory and induce insulin resistance.

JNK-1, -2, and -3 are members of the MAPK family of proteins that control transcription via AP-1 proteins c-Jun and JunB (89). JNK activity is increased in the liver, adipose tissue, and muscle of obese rodents and humans, and whole-body JNK knockout mice are protected against insulin resistance (180, 493). Specifically, JNK activation results in Ser307 phosphorylation of IRS-1 blocking insulin signal transduction (5, 6, 128, 363). Although adipose tissue specific JNK

knockout protects against diet-induced insulin resistance (419), the direct role of JNK as a cause of insulin resistance has been questioned as the use of hepatic and skeletal muscle specific knockouts of JNK has yielded different results from the adipose tissue knockout model (419, 420, 428). Thus, JNK activation may have tissue specific roles in the puzzle of insulin resistance and the blockade of JNK activity specifically in the adipose tissue may yield the greatest rewards.

1.2.4.2. Altered lipid metabolism and the generation of fatty acid derivatives.

Altered lipid metabolism has become well established in the development of insulin resistance. It has been known for some time that circulating free fatty acids (FFAs) can induce insulin resistance and lowering circulating FFAs improves insulin resistance (44, 240, 432). The association with insulin sensitivity is stronger between intramuscular lipid content than circulating FFA concentration (268), and intramuscular lipid storage has become a heavily researched field in the context of insulin resistance. The proper functioning of the adipose tissue to sequester fatty acids plays a role in whole-body lipid metabolism and prevents inappropriate lipid storage in the skeletal muscle and liver (149). It should be noted that exercise uses intramuscular lipids and that athletes store equal to or even greater amounts of lipids in the skeletal muscle compared to type 2 diabetics despite being insulin sensitive, a phenomenon now known as the ‘athlete’s paradox’ (143).

As a great deal of lipid oxidation occurs in the skeletal muscle, mismatches between lipid and glucose oxidation may result in the generation of fatty acid derivatives (341). Feeding a high-fat diet to rodents actually increases skeletal muscle mitochondrial biogenesis, likely as a means to increase oxidative capacity to handle excess dietary lipids (161, 495). This

compensatory mechanism leads to differential increases in components of fatty acid metabolism resulting in a mismatch between fatty acid β -oxidation with the downstream TCA cycle and oxidative phosphorylation. This mismatch leads to increased generation of acetyl-CoA, long-chain acyl-CoAs and acyl-carnitines, DAGs, ceramides, and phosphatidic acid, which activate pathways leading to inhibited insulin signaling.

As lipid excess builds up, acetyl-CoA and acyl-CoAs from β -oxidation accumulate and disrupt mitochondrial and cytosolic metabolism, and the generation of acyl-carnitines that are readily exported from the mitochondria, further disrupt cellular metabolism and contribute to insulin resistance (261, 339). Elevation of these metabolites, DAGs, and ceramides via pharmacological and genetic methodologies induces insulin resistance, and inhibition of the generation of DAGs and ceramides restores insulin sensitivity (181, 342, 428). DAGs activate members of the PKC family of molecules that inhibit insulin signaling (342, 428) by phosphorylating IRS-1/2 on multiple serine residues and decreasing tyrosine phosphorylation (164, 214, 229). Ceramides are sphingolipids that inhibit insulin signaling by impairing Akt2 activation (428, 439). Thus, DAG and ceramide accumulation may be a powerful mechanism to inhibit insulin signaling but their inhibition would be difficult due to their varying roles in multiple tissues.

1.2.4.3. Mitochondrial abnormalities. At the heart of the above-mentioned alterations in lipid metabolism is the mitochondria. The mitochondria are double membraned organelles that regenerate ATP by oxidizing carbon substrates (276, 376). The mitochondrial matrix contains the enzymatic machinery of the TCA cycle and β -oxidation which provide reduced NADH and FADH₂ to the electron transport chain (ETC) which creates the proton

gradient across the inner mitochondrial membrane (376). The ETC complexes (I-IV) shuttle electrons from donors (NADH at complex I and FADH₂ at complex II) to a final electron acceptor, oxygen, to form water at complex IV. This process results in transport of protons from the matrix to the intermembrane space creating an electrochemical gradient across the inner membrane that is coupled to ATP production by moving protons back into the matrix via mitochondrial ATPase (complex V) (376). To match energy demands, changes in mitochondrial enzyme content and mitochondrial density are coordinated by the mitochondrial transcription factor, mtFAM, and by nuclear coactivators such as peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), PGC-1 β , and peroxisome proliferator-activated receptor γ coactivator-related 1 (PPRC1) (376, 531).

A number of published reports have linked the susceptibility to developing insulin resistance and type 2 diabetes to aberrant skeletal muscle mitochondrial functionality, reduced activity of oxidative phosphorylation and PGC-1 α expression in type 2 diabetic patients (238, 260, 327, 375, 387, 388, 406). These traits are also present in offspring of diabetic parents and in glucose tolerant monozygotic twins of diabetic patients (30, 388). These deficits can be repaired by lifestyle interventions, especially weight loss and exercise, and the improvements in mitochondrial mass correlate with improvements in insulin sensitivity (314, 390, 479, 480). Thus the reductions in mitochondrial mass and functionality may be a result of environmental conditions (e.g., physical inactivity and caloric excess) rather than ingrained in one's genetic makeup alone.

The mitochondrial role in insulin resistance has shifted towards an understanding of metabolic flexibility. Metabolic inflexibility represents inappropriate physiological and cellular

fuel selection during periods of rest or stress. Kelley & Simoneau showed that obese diabetics had lower activity of mitochondrial enzymes during fasting, but increased activity of glycolytic enzymes. Along with these observations, in leg balance studies, diabetics had lower lipid oxidation when fasting (higher RQ) and an inability to switch to carbohydrate utilization during the post-prandial state or upon insulin stimulation. These traits were subsequently termed “metabolic inflexibility” (239, 376), and this disruption in whole-body metabolism has been linked to inappropriate substrate selection at the mitochondrial level (339).

1.3. HEAT SHOCK PROTEINS & THE HEAT SHOCK RESPONSE

The induction of the heat shock response was first described by Italian scientist Ferruccio Ritossa when an inadvertent application of heat was applied to *Drosophila* salivary glands by accidental incorrect incubator settings (9, 175, 288, 405). The heat shock led to chromosomal puffing that was later characterized as increased transcription leading to increased protein translation of a specific family of proteins later coined heat shock proteins (HSPs). The heat shock response is a classical stress response that detects, monitors, and responds to environmental cues and changes, in its simplest form temperature, and then modifies the intracellular environment to compensate for the environmental changes (329).

It has become clear that the activation of the heat shock response, or constitutive overexpression of the HSPs themselves, is protective against cell death from a variety of stressors (329). The HSPs produced as a result of heat stress directly assist in thermotolerance and protection against further stress. This protection is extended to a number of other conditions

in addition to heat including (i.e., ethanol, hypoxia, sodium arsenite, and cadmium chloride), which condition against successive heat stress, and vice versa, prior heat stress protects against these stressors (287, 288, 391, 502).

The induction of HSPs *in vivo*, specifically HSP72, occurs in most mammals and humans following heat stress in skeletal muscle, cardiac muscle, liver, lungs, brain, different regions and cells of the central nervous system, adrenal glands, spleen, lymphocytes, small intestine, bladder, and in this dissertation, adipose tissue (54, 81-83, 105, 118, 125, 154, 155, 206, 230, 265, 266, 295, 300, 301, 323, 351, 410, 412, 425, 438, 452, 508, 509). Exercise is also a potent activator of the heat shock response (297). The physiological and cellular sensors responsible for activating the heat shock response have not been fully elucidated, but unfolded or misfolded proteins, protein aggregation, and ER stress have been described as the major culprits (9, 329). Thus, protein damage caused by either heat shock itself or other factors resulting in protein unfolding result in activation of heat shock factor-1 (HSF-1), the primary transcription factor responsible for the induction of the heat shock response.

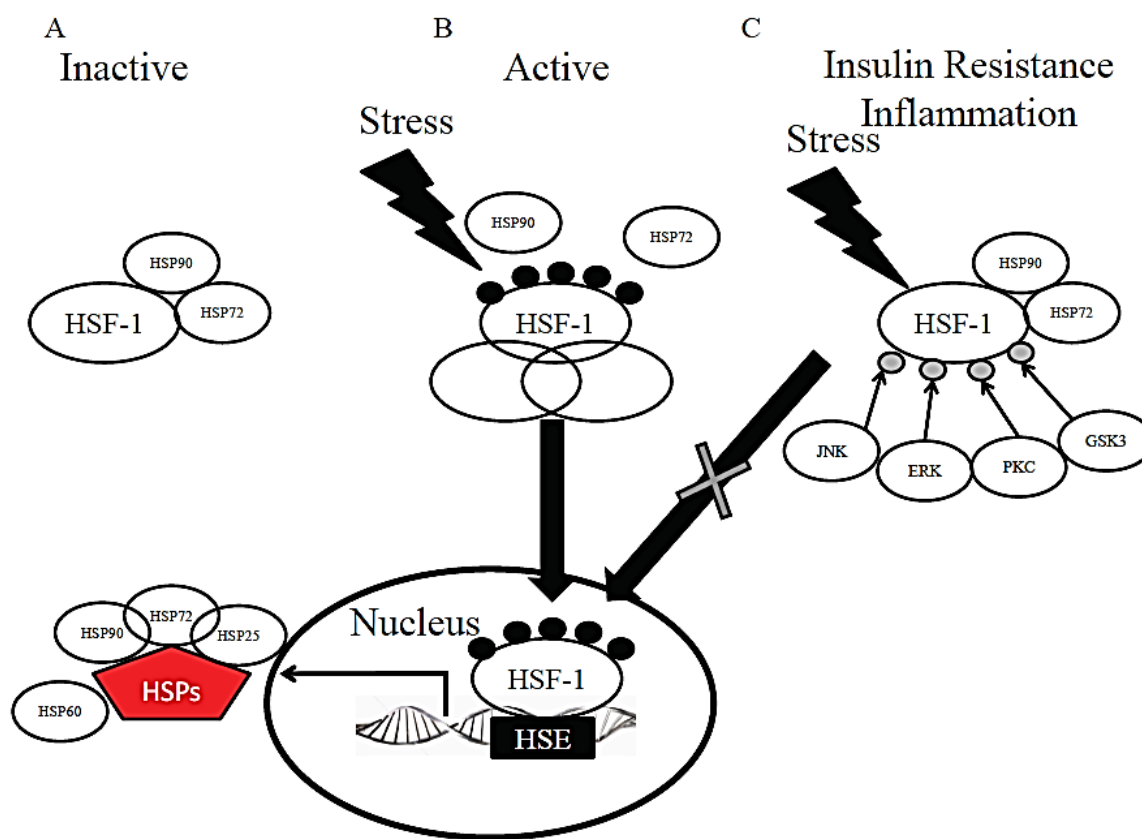


Figure 2. The heat shock response. (A) Unstressed, HSF-1 is held in the cytosol as an inactive monomer by transient binding of HSP72 and HSP90. (B) Upon application of stress, HSP72 and HSP90 dissociate from HSF-1, and HSF-1 trimerizes, becomes hyperphosphorylated, and translocates to the nucleus where it binds heat shock response (HSEs) in the DNA increasing transcription of HSPs. (C) During insulin resistance and inflammation, the heat shock response is blunted due to inhibitory phosphorylation of HSF-1 by JNK, ERK, PKC, and GSK3.

1.3.1. Heat shock factor-1. HSF-1 is a winged helix-turn-helix transcription factor that upon activation translocates to the nucleus and binds to heat shock response elements (HSE) in the promoter regions of heat responsive genes resulting to the induction of HSP gene transcription (329, 371, 380, 529). HSF-1 is expressed in most tissues and cell types (116), and is the master regulator of the heat shock response as HSF-1 is generally required for stress-induced increases in HSPs (290, 312, 328, 504, 530). However, HSPs are still constitutively expressed in HSF-1 null cells and animals (211, 312, 352, 533, 534, 543, 544). In addition, HSF-1 plays a role in development, life span and aging, immune responses, circadian rhythms, and some malignant cancers.

HSF-1 is regulated by multiple protein-protein interactions, post-translational modifications, and subcellular localization (9). HSF-1 exists in the cytosol as an inactive monomer prior to the application of stress by transient binding of HSP90 and HSP70 (**Figure 2A**) (12, 396, 547). Upon application of stress, HSF-1 becomes an active trimer, hyperphosphorylated on multiple serine residues including Ser230 and Ser326 (77, 148, 172, 186, 434), and translocates to the nucleus and binds HSEs in the DNA (**Figure 2B**) (23, 547). HSF-1 nuclear localization and DNA-binding can also be inhibited by phosphorylation on several serine residues by stress kinases such as JNK, extracellular signal-regulated kinase (ERK)-1/2, GSK-3, PKC α , and PKC ζ which lead to HSF-1 sequestration to the cytosol (**Figure 2C**) (65, 66, 86, 369, 513). In negative feedback fashion, increased levels of HSP90 and HSP70 following heat stress inhibit continued HSF-1 activation by blocking trimerization and nuclear translocation (337, 396, 445). HSF-1 DNA binding is also inhibited by acetylation by p300-CREB binding protein (CBP), and HSF-1 is deacetylated by the NAD⁺-dependent sirtuin 1 (Sirt1), which promotes longer DNA binding (521).

1.3.2. Heat shock proteins. In general, the HSPs function as molecular chaperones that aid in proper folding, refolding, and transport of newly translated proteins or proteins that become unfolded (334). HSPs are generally arranged into different families based upon molecular weight: 1) small HSPs (8-27 kDa), 2) HSP60 (60 kDa), 3) HSP70 (70-73 kDa), and HSP90 (90 kDa).

1.3.2.1. Small heat shock proteins. HSP25/27 (herein referred to as HSP25) has a number of cellular functions not limited to its role as a molecular chaperone including microfilament stabilization, the glutathione system, signal transduction, growth and differentiation, and protects against oxidative and thermal stress (32, 107, 126, 204, 205, 274, 280, 281, 325, 437, 516). α B-crystallin (20 kDa), another small molecular weight HSP, is from the family of crystallin proteins found in mammalian eye lenses (334). HSP25 and α B-crystallin share great sequence similarity (91, 320), directly associate with one another (234), share common cellular responsibilities, and both are induced in response to heat and oxidative stress (169, 210, 233). Like HSP25, α B-crystallin has also been shown to be involved in cytoskeletal stabilization and together HSP25 and α B-crystallin appear to be important for skeletal muscle recovery from exercise and protection against atrophy by stabilizing components of the sarcomere (18, 100, 254, 377).

1.3.2.2. Heat shock protein 60. HSP60 is a nuclear encoded mitochondrial chaperone that is essential for proper folding and assembly of proteins imported into the mitochondria (95, 187, 334). HSP60 is essential for proper mitochondrial function as proteins imported into the mitochondrial matrix directly interact with HSP60 (166, 275, 299) and deficits in HSP60 result in disease phenotypes. In humans, a few rare genetic disorders are associated

with HSP60 deficiency including congenital lactic acidemia (4, 47). Homozygous deletion of HSP60 in mice is lethal; however, heterozygous mice display an insulin resistant, pro-inflammatory phenotype with mitochondrial deficits (63, 247). Intracellular and extracellular location of HSP60 likely drives many of its roles. Although the majority of HSP60 is located in the mitochondria of cardiomyocytes (~80%), there is a fraction of HSP60 located extramitochondrially (~20%) that is anti-apoptotic (151). Extracellular HSP60 is pro-inflammatory and HSP60 can be released from adipose tissue activating inflammatory pathways in adipocytes and skeletal muscle (302, 303). HSP60 is increased by heat stress and other stresses that induce the heat shock response such as exercise (334, 348, 489).

1.3.2.3. Heat shock protein 70/72. HSP72 is the most highly conserved HSP between species with human sharing 96.6%, 73%, and 60% amino acid sequence similarity with rodent HSP72, *Drosophila* HSP72, and bacterial DnaK, respectively (288, 404, 507). There are four members of the HSP70 family, two cytosolic forms, a constitutive/cognate form (HSC70, 73 kDa) and an inducible form (HSP72, 72 kDa), as well as the ER chaperone glucose-regulated protein 78 (GRP78 or Bip, 78 kDa), and a mitochondrial chaperone HSP75 (mtHSP70, also known as mortalin, 75 kDa) (294).

HSP72 is expressed by two genes: *hspa1a* and *hspa1b*. The protein contains an N-terminal ATPase domain, which is necessary for its chaperone functions, a C-terminal protein binding domain, and a linker domain connecting the two (356, 423). A number of co-chaperones (i.e., DnaJ/HSP40, Bag1, Hip) are present with HSP72 and assist in its chaperone function (95, 356). Although HSP72 is best described for its role as a molecular chaperone, HSP72 interacts with a number of cell signaling pathways, is involved in mRNA stabilization and degradation,

assists in protein degradation, helps to regulate cell death, and has been described to deliver substrate proteins to translocases that transport these proteins across organelle membranes (423, 539). Recently, HSP72's role in insulin resistance, mitochondrial function, and type 2 diabetes has been partially characterized and will be described below.

1.3.2.4. Heat shock protein 90. HSP90 is involved in folding substrate proteins (i.e., protein kinases), as well as activation of transcription factors, steroid hormone receptors, and oncogenic proteins (i.e., tumor suppressor p53) (423, 516), but HSP90 also plays a role in cell signaling and cell death pathways including chaperone-mediated autophagy (98). HSP90 levels are high in the cytosol and are further increased upon stress (517). HSP90 plays an important role in inhibiting constitutive HSF-1 activity, along with HSP72, by preventing HSF-1 trimerization and translocation to the nucleus. This function makes HSP90 an attractive target for inhibition to elicit a heat shock response pharmacologically.

1.3.3. HSPs in the primary metabolic tissues. Much of the research surrounding HSPs and the induction of HSPs has been performed in skeletal muscle, but less has been performed in the liver and adipose tissue. These tissues are extremely important for maintaining whole-body glucose homeostasis and insulin sensitivity, but have different roles in the progression of insulin resistance and type 2 diabetes. HSPs, specifically HSP72, have been shown to play a protective role in the development of insulin resistance and type 2 diabetes primarily in skeletal muscle, but few studies have examined the role of HSP72 in the liver and adipose tissue.

1.3.3.1. HSPs in the skeletal muscle. Most of the work regarding HSP expression, especially as it relates to insulin resistance, has been performed in skeletal muscle. HSP levels are greater in more metabolically active muscle fibers. We and others have observed the

constitutive HSP72 and HSP60 levels are greater in red, oxidative skeletal muscle compared to white, glycolytic skeletal muscle (45, 152, 278, 296, 361). Furthermore, the induction of HSPs following heat treatment is greater in glycolytic muscle where constitutive levels are low. HSP25 and α B-crystallin are also more highly expressed in cardiac muscle and oxidative skeletal muscle fibers compared to glycolytic muscle fibers (202, 209, 350).

Muscle fiber type is often related to the predominate expression of a particular isoform of myosin heavy chain (MHC) within a muscle fiber. Based in this, there are three basic muscle fiber types in skeletal muscle of humans: type I, IIa, and IIx; and a forth fiber type is expressed in rodents that is not expressed in humans (IIb). Oxidative skeletal muscle contains a greater preponderance of type I fibers, and glycolytic skeletal muscle contains a greater preponderance of type II fibers. In general, type I muscle fibers generate less force and fatigue more slowly when stimulated to contract repeatedly compared to type II muscle fibers. Oxidative muscles contain greater content of myoglobin, oxidative enzyme content, and greater mitochondrial density while glycolytic muscles contain less myoglobin, greater content of glycolytic enzymes, and fewer mitochondria.

In humans, most muscles are mixed and contain approximately 50% type I fibers and 50% type II fibers. In rodents, particular muscles contain a large content of a particular muscle fiber type making it easier to study particular muscle fiber types without dissecting individual muscle fibers. For instance, the soleus of a rat contains approximately 84% type I fibers, 7% type IIa fibers, and 9% type IIx fibers while the extensor digitorum longus (EDL) contains 4% type I fibers, 20% type IIa fibers, 38% type IIx fibers, and 38% type IIb (13, 94). Of particular importance for type 2 diabetes is that diabetic patients undergo a shift from type I, oxidative

muscle fibers to expressing a greater number of type II, glycolytic muscle fibers (178, 474). The opposite occurs with endurance training, where there is a shift towards a greater number of type I oxidative muscle fibers (457).

One of the most widely described treatments for insulin resistance and type 2 diabetes is exercise (2). Acute exercise increases skeletal muscle lipid oxidation and increases insulin sensitivity for 24-48 hr (167, 183, 424, 440, 526, 545). In addition to its influence at the whole-body and cardiovascular levels, chronic endurance exercise increases mitochondrial enzyme activity and mitochondrial density in multiple tissues (120, 140, 182, 185, 467), modifies skeletal muscle fiber type towards a more oxidative phenotype (88, 140), and increases skeletal muscle capillary density (279). The reverse of these adaptations is observed during physical inactivity, a lifestyle undertaken by many insulin resistant individuals.

For a number of years it has been known that exercise, which typically results in a transient increase in body and muscle temperature (48), increases HSP transcription and protein levels. A single bout of aerobic exercise increases HSP72 transcription and expression in skeletal muscle of humans and rodents (60, 112, 177, 242, 245, 295, 335, 353, 359, 372, 373, 395, 425, 449, 452, 506), but also in the myocardium (245, 297, 316-318, 324, 353, 425, 452), blood vessels (324, 449), liver (142, 265, 425), brain (509), and multiple other tissues. HSP60 levels increase in skeletal muscle following endurance exercise. This increase in HSP60 precedes any changes in mitochondrial protein content, but coincides with increased mitochondrial enzyme activity and levels of positive regulators of mitochondrial biogenesis (187, 242, 295, 335). Chronic endurance training also results in increased HSP levels and levels of HSF-1 (141, 177, 244, 317, 345, 359, 395, 426, 459). An important aspect to the increase in HSPs is the question

as to whether these increases are due to temperature or contractile activity. Muscle contractions in the absence of rises in body and muscle temperature increase HSP levels (245, 336, 452), indicating that molecular mechanisms independent of temperature can induce the heat shock response.

A number of potential mechanisms have been explored besides temperature that explains increases in HSPs levels following exercise. These mechanisms include hypoxia (31), glycogen depletion (113), oxidative stress (110, 117), energy depletion/ATP pools (334), mechanical damage (144), decreased pH (338), calcium ion influx (338), and increased circulating catecholamines (372). An attractive hypothesis would be that calcium ions induce the heat shock response as calcium ion concentration increases in the cytosol to initiate muscle contractions and is a powerful activator of a number of signaling pathways that induce mitochondrial biogenesis and influence metabolism (i.e., AMPK). As skeletal muscle metabolic health is intimately tied to the development of type 2 diabetes, the role of inducible HSPs in insulin resistance is important.

1.3.3.2. Adipose tissue dysfunction and the role of HSPs in white adipose tissue.

Once thought to be a benign tissue that's only responsibility was to store fatty acids to be released when called upon by other organs in stress, the adipose tissue is now known to play a far larger role in whole body physiology and insulin sensitivity (43, 540). Adipose tissue is important for thermoregulation and for protection of vital organs, and is also well recognized as an endocrine organ. Since the characterization of leptin, adipose tissue has been found to secrete over 600 adipokines and hormones, many of which are pro-inflammatory and can themselves induce insulin resistance, including adiponectin, retinol binding protein 4 (RBP4), interleukin-6 (IL-6), IL-1 β , and TNF α . Now with an abundance of nutrition available for consumption, obesity

(or increased adiposity) has become a large problem in the modern world. Although obesity is associated with many co-morbidities, its distribution within the body may be of greater importance.

Adipose tissue has different forms, including white (WAT) and brown adipose tissue (BAT), and a newer described beige adipose tissue. WAT is dispersed into different depots in mammals with prominent SCAT and abdominal or visceral WAT depots appearing in humans. In male rodents, there is also a large epididymal WAT (eWAT) depot, which is largely absent in humans that are commonly studied in rodents as a model of abdominal WAT. Visceral WAT content has been correlated with risk of type 2 diabetes and CVD while SCAT is thought of as protective against metabolic disease as implantation of SCAT into the visceral compartment improves insulin sensitivity (415, 485). These depots have different intrinsic metabolic activities as adipocytes from abdominal depots have greater secretion of inflammatory cytokines (108, 207, 385, 505), greater lipolysis and fatty acid re-esterification (14, 170, 277, 481), greater oxygen consumption and mitochondrial protein content (96, 264), but lower insulin sensitivity (546). HSP levels are greater in the visceral WAT compared to other WAT depots, but this is complicated by HSP content in other components of the adipose tissue such as the stromal-vascular fraction (SVF) (379, 383, 421, 458).

During obesity and caloric excess, triglyceride storage increases in the WAT, especially in the SCAT depots. In susceptible individuals, when the SCAT becomes overloaded, fatty acid storage is redistributed into the visceral depots as well as into the skeletal muscle, cardiac muscle, liver, and pancreas contributing to whole-body and hepatic insulin resistance (43, 149, 415). The liver triglyceride content is intimately tied to the visceral WAT as the release of pro-

inflammatory cytokines and fatty acids from the visceral WAT enters the portal circulation prior to the systemic circulation bathing the liver in exaggerated concentrations of these molecules.

During obesity and insulin resistance, the adipocytes themselves become dysfunctional as the WAT itself becomes inflamed. Adipocytes hypertrophy to increase the capacity to store triglycerides, but larger adipocytes have greater basal lipolytic rates and are less insulin sensitive than smaller adipocytes reducing proper fatty acid uptake and storage (15, 106). Increases in capillarity cannot keep pace with increased cell size to supply sufficient oxygen levels leading to hypoxia of the adipocytes, especially in the SCAT, where increased capillarization cannot keep pace with hypertrophy (43, 133, 374, 486). Hypoxic, dysfunctional adipocytes release chemoattractant molecules including monocyte chemoattractant protein-1 (MCP-1), chemerin, progranulin, and monocyte colony stimulating factor-1 (CSF-1) (43, 227, 435). These molecules recruit macrophages to the adipose tissue which release pro-inflammatory cytokines (80, 165, 515, 532) causing the adipocytes themselves to become dysfunctional and secrete their own pro-inflammatory cytokines/adipokines (149). These pro-inflammatory cytokines activate lipolysis, reduce triglyceride storage, and disrupt peroxisome proliferator-activated receptor- γ (PPAR γ) expression, a transcription factor essential for proper adipose tissue function, leading to redistribution of fatty acid storage from the SCAT into the visceral WAT and finally into other tissues such as skeletal muscle (149). Hyperinsulinemia results in a loss of insulin's inhibition of lipolysis, also contributing to the release of fatty acids into the circulation.

The role of HSPs in the adipose tissue has not been well described. We previously observed that HSP72 is induced in the eWAT of heat treated rats(154). Elsewhere, it has been reported that six wk of voluntary wheel running does not to increase HSP72 levels in WAT in

the absence of other stress (i.e., tail shock) (458). Cold exposure has been shown to increase HSP levels in BAT and this appears to be regulated by α -adrenergic receptor activation (306-309). Despite skeletal muscle HSPs being implicated in the development and protection type 2 diabetes, few reports have explored WAT HSPs as it relates to diabetes. One report did find that although HSP72 expression in the skeletal muscle was positively associated with insulin sensitivity, this association was not found in the adipose tissue (174).

1.3.3.3. HSPs in the liver and NAFLD. As briefly mentioned previously, HSPs or heat treatment have been shown to protect the liver from injury, but much of this research has been centered upon damage induced by toxins and ethanol. Very little work has been performed in the liver regarding what role HSPs play in metabolic syndrome and insulin resistance. NAFLD is the increase in hepatic triglyceride accumulation in the absence of excess alcohol consumption (> 20 g/d for men, > 10 g/d for women) (49), and is strongly associated with obesity, metabolic syndrome, and type 2 diabetes. The prevalence of NAFLD in the U.S. adult population is approximately 30% and is particularly high in obese patients ($>75\%$) (16, 201, 222).

NAFLD is the hepatic manifestation of metabolic syndrome and encompasses a range of severity from simple hepatic steatosis, to non-alcoholic steatohepatitis (NASH), to fibrosis, and finally cirrhosis (16, 75, 401, 477). Increased triglyceride deposition occurs as a result of an imbalance between lipid accumulation and lipid disposal (326, 343). Lipid accumulation occurs as lipids are taken up from the circulation in the form of FFAs and dietary lipoproteins, but lipids also accumulate as a result of *de novo* lipogenesis. Lipid disposal occurs in the form of very-low density lipoproteins (VLDL) release and β -oxidation of fatty acids in the mitochondria (326, 343). Hepatic insulin resistance occurs early in the progression of type 2 diabetes possibly

preceding skeletal muscle insulin resistance. This results in a blunted ability of the liver to reduce glucose output following a meal contributing to post-prandial glucose intolerance.

Low levels of physical activity have been implicated in the development of NAFLD (194, 282, 386), and exercise has been touted as a treatment option for NAFLD (225, 402, 403, 498). In addition, low levels of CRF have been shown to be inversely associated with the prevalence of NAFLD (70, 228, 263, 313). HSP72 expression in the liver has been negatively associated with severity of NASH (97). Exercise can induce HSP72 expression in the liver, which may play a protective role in the face of hepatic insulin resistance (142, 265, 425). Improved mitochondrial function plays a role in the protective effects of exercise as decreased mitochondrial function has been implicated in the development of NAFLD in animal models of obesity or low CRF (402, 476).

1.3.4. Heat shock proteins protect against insulin resistance in the skeletal muscle, liver, and adipose tissue. For centuries, treating diabetics with ‘healing hot waters’ was popular in Eurasia (189). What is known about the relationship between HSPs and insulin resistance/type 2 diabetes has grown throughout the last 15 years. The seminal recent work in this field occurred in 1999 with the publication of a small study by Dr. Philip Hooper in the *New England Journal of Medicine*. This small study showed that 30 min hot tub treatments, 6 d/wk, for 3 wk could significantly improve diabetic symptomology in type 2 diabetic patients (188). Since this report, a number of reports have described reduced HSP levels or blunted induction of HSPs following stress in diabetic patients and other models of diabetes. In addition, it has been shown that an increase in HSP levels can improve glucose-insulin homeostasis and insulin sensitivity.

Kurucz *et al.* and Patti *et al.* were the first to report that HSP72 mRNA levels were lower in the skeletal muscle of type 2 diabetics compared to non-diabetics, and HSP72 mRNA levels positively correlated with insulin sensitivity (269, 375). Elsewhere, lower HSP72 mRNA or protein levels in skeletal muscle of human diabetics and animal models of diabetes have been reported, including reduced expression in the liver, heart, and adipose tissue (17, 50, 67, 97, 237, 283, 382, 473). The protein levels of HSF-1 are also lower in the skeletal muscle of diabetic humans and rats (17, 411). In the adipose tissue, the expression HSP72 in obese and type 2 diabetic individuals is complicated. Perez-Perez *et al.* reported that HSP levels are higher in obese non-diabetics but lower in obese type 2 diabetics compared to lean non-diabetics (382, 383), and this has been confirmed elsewhere that HSP levels are increased in obese non-diabetics (478). Little is known about how the WAT function is influenced directly by HSPs or heat treatment.

The induction of HSPs appears to be blunted in diabetic models as well. Atalay *et al.* showed that the exercise induced increases in skeletal muscle HSP72 and HSF-1 levels were lower in STZ-induced diabetic rats compared to controls (17). In diabetic monkeys, the activation of HSF-1 is lower when liver samples are exposed to heat stress (235). In contrast, Najemnikova *et al.* observed that the induction of HSP72 and HSP25 was greater in the heart, kidney, and liver of heat-treated STZ-induced diabetic rats compared to controls. Differences between these two studies in diabetic rodents included the length of time following STZ treatment, age, and strain of rats. HSP72 can be glycosylated, which inhibits its function (28), thus the duration of diabetes and poorly controlled glucose levels may play a role reducing the function and induction of HSP72. More work is needed to determine the mechanisms behind the

blunting of the heat shock response in diabetes. Whether the induction of HSPs is blunted in WAT of diabetics or whether the induction is different between WAT depots has not been tested.

The induction of HSP72 and *in vivo* heat treatments that increase HSP72 levels have been touted as a viable treatment option for insulin resistance and type 2 diabetes. In addition to Hooper's seminal work, *in vivo* heat treatment has been shown by our laboratory and others to improve whole-body glucose-insulin homeostasis and lipid profile, increase skeletal muscle insulin sensitivity, and reduce adipose tissue mass (3, 28, 67, 154, 188, 256). Transgenic overexpression of HSP72 in the skeletal muscle improves insulin sensitivity, reduces adipose tissue mass, increases mitochondrial content and activity of mitochondrial proteins (67, 173). Pharmacological induction of HSP72 using geranylgeranylacetone (GGA) improves hyperglycemia, reduces liver and muscle JNK activation, improves hepatic insulin signaling, and reduces adipose tissue mass (3, 235). Using a model of heat stress combined with mild electrical shock (MES), another group has reported improvements in whole-body insulin resistance, reduced hepatic JNK activation and hepatic fat content, increased hepatic insulin responsiveness, reduced adipose tissue mass, and reduced β -cell apoptosis and ER stress in mice fed a high-fat diet (330). In humans, few trials have been performed using heat treatment. In one trial, sauna treatment (20 min, 3x/wk, for 3 mo) improved quality of life scores in diabetic patients (29), and in another repeated sauna treatments (60°C, 15 min) reduced body weight and body fatness in obese individuals while improve overall hemodynamics (37). Other trials have investigated sauna treatment for its role in improving endothelial function in patient populations with cardiovascular disease (208, 243). Thus, while more work is needed in human models, heat treatment improves diabetic symptomology.

From a mechanistic standpoint, HSPs have multiple physiological roles making their true beneficial role in different tissues elusive. Our laboratory has focused on the anti-inflammatory role of HSPs in skeletal muscle insulin sensitivity. HSP72 has been shown to bind to and inhibit JNK activation, and HSP25 has been shown to inhibit IKK β activation, both of which inhibit insulin signaling when active (368, 370). Previously, we showed that weekly *in vivo* heat treatments reduced JNK and IKK β activation in the skeletal muscle in rats fed a HFD and insulin resistant aged rats (154, 155). Others have shown that heat treatment or GGA administration reduces JNK activation in the skeletal muscle and liver (3, 330). We also showed that lipoic acid, an HSP co-inducer, reduced JNK and IKK β activation in the skeletal muscle in rats fed a HFD (153). Febbraio & colleagues have shown that transgenic overexpression of HSP72 in the skeletal muscle and *in vivo* heat treatment of mice fed a HFD reduced JNK activation, as did administration of BGP-15 another HSP co-inducer, to *ob/ob* mice (67, 173). This same group has recently shown that incubation of muscle with palmitate to activate JNK was not lower in HSP72 overexpressing mice (173). Thus, the role of HSP72 in inhibiting JNK activation may be situationally dependent.

Another potential mechanism by which heat treatments and increased HSP72 levels improve glucose homeostasis is by increasing mitochondrial biogenesis and/or function. As defective mitochondrial function has been implicated in the pathology and etiology of type 2 diabetes, this is of potential therapeutic relevance. Our laboratory has shown that weekly *in vivo* heat treatments increase activity of mitochondrial enzymes in skeletal muscle while reducing PGC-1 α levels, and that heat treatment of L6 cells increased mitochondrial fatty acid oxidation (154). The reduction in PGC-1 α levels may indicate a reduced requirement for mitochondrial biogenesis due to improved functionality. Transgenic overexpression of HSP72 in the skeletal

muscle increases mitochondrial enzyme activity, mitochondrial content, and increases running capacity nearly two-fold (67, 173). Elsewhere, Chen *et al.* and Tamura *et al.* have observed that heat stress increases mitochondrial enzyme activity *in vivo* (59, 471). Furthermore, Liu & Brooks have found that heat treatment of C2C12 myotubes increased mitochondrial biogenesis by transiently activating AMPK leading to increased content of mitochondrial respiration chain complexes after repeated heat treatments (293). An increase in the expression of HSP72 may improve mitochondrial oxidative capacity by modulating mitophagy.

Recently, HSP72 has been shown to be involved in regulating autophagy/mitophagy and this may provide a mechanism for the role of HSP72 in mitochondrial functionality and oxidative capacity. Protein degradation occurs in eukaryotic cells by – 1) the ubiquitin-proteasome pathway and 2) the autophagy-lysosomal pathway. When a protein or organelle is targeted for degradation by the autophagy-lysosomal pathway, a double membraned autophagic vacuole is formed called an autophagosome which merges with the lysosome to form an autolysosome and the autophagosome is degraded by the lysosomal enzymes (397, 429). Defects in the autophagic processes have been implicated in liver failure, neurodegenerative diseases, muscle atrophy, and metabolic syndrome (397, 470). Recently, it has been shown that proper autophagy is essential for glucose homeostasis (168). Several studies have now shown that acute exercise and exercise training increase markers of autophagy in the skeletal muscle (168, 215-217, 246, 289). In addition, autophagy has been shown to regulate hepatic lipid metabolism (53, 451). In a process known as lipophagy, autophagy breaks down lipid droplets to mobilize fatty acids potentially for oxidation in the mitochondria (397, 451). Thus, proper autophagy is likely necessary to mobilize triglycerides from lipid droplets, but also for proper formation of the lipid droplets.

Mitophagy is the autophagic process of clearing damaged and/or depolarized mitochondrial. Defective mitophagy is a cause of a number of diseases. Pink1 and Parkin regulate Mitophagy, and mutations in these proteins result in recessive familial Parkinson's disease (99, 333, 537). Pink1 is recruited and docks on dysfunctional mitochondria recruiting the E3 ubiquitin ligase Parkin, which polyubiquinates the outer mitochondrial membrane targeting it for degradation (99). HSP72 has been shown to associate with Parkin and facilitate Pink1 binding allowing for the mitochondria to be targeted for degradation through mitophagy. HSP72 knockout mice have increased levels of Parkin, but the ability to degrade mitochondria is hampered and dysmorphic, dysfunctional mitochondria accumulate. Thus, the autophagy-mitophagy pathway may be influenced by HSP72 and heat treatments maintaining a greater pool of functional mitochondria.

1.4. RESEARCH QUESTIONS

In lieu of our previous experiments showing that the induction of HSP72 following *in vivo* heat treatment improves whole-body insulin glucose-insulin homeostasis, skeletal muscle insulin sensitivity, and reduces epididymal fat pad mass in high-fat fed rats, it is necessary to perform a number of follow-up experiments. First, to investigate the role of HSPs as well as acute and chronic heat treatment on WAT HSP levels and WAT fatty acid handling. Second, we will determine whether heat treatment or the induction of HSPs plays any role in protection against hepatic steatosis. Finally, in a model of low intrinsic aerobic capacity, we will investigate whether constitutive HSP levels and the induction of HSPs are different between rats with high and low endurance running capacities, and whether heat treatment improves skeletal muscle

insulin signaling and hepatic steatosis in a model of lower aerobic capacity. The answers to these questions will further advance the role of HSPs, especially HSP72, as an alternative treatment modality of metabolic diseases by expanding their protective role beyond the skeletal muscle.

CHAPTER 2

HEAT SHOCK PROTEINS: IN VIVO HEAT TREATMENTS REVEAL ADIPOSE TISSUE DEPOT-SPECIFIC EFFECTS

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2.1. ABSTRACT

Heat treatments (HT) and the induction of heat shock proteins (HSPs) improve whole-body and skeletal muscle insulin sensitivity while decreasing white adipose tissue (WAT) mass. However, HSPs in WAT have been understudied. The purpose of the present study was to examine patterns of HSP expression in WAT depots, and to examine the effects of a single *in vivo* HT on WAT metabolism. Male Wistar rats received HT (41°C, 20 min) or sham treatment (37°C), and 24 hr later subcutaneous, epididymal, and retroperitoneal WAT depots (SCAT, eWAT, and rpWAT, respectively) were removed for *ex vivo* experiments and Western blotting. SCAT, eWAT, and rpWAT from a subset of rats were also cultured separately and received a single *in vitro* HT or sham treatment. HSP72 and HSP25 expression was greatest in more metabolically active WAT depots (i.e., eWAT and rpWAT) compared to the SCAT. Following HT, HSP72 increased in all depots with the greatest induction occurring in the SCAT. In addition, HSP25 increased in the rpWAT and eWAT, while HSP60 increased in the rpWAT only *in vivo*. FFA release from WAT explants was increased following HT in the rpWAT only, and fatty acid re-esterification was decreased in the rpWAT but increased in the SCAT following HT. Heat treatment increased insulin responsiveness in eWAT, but not in SCAT or rpWAT. Differences in HSP expression and induction patterns following heat treatment further supports the growing body of literature differentiating distinct WAT depots in health and disease.

Keywords:

HSP72, metabolism, stress response, lipolysis, fatty acid re-esterification

2.2. INTRODUCTION

We previously demonstrated that *in vivo* heat treatments improve whole-body glucose homeostasis and insulin-stimulated glucose uptake in skeletal muscle (154, 155). Heat treatments increased HSP72 expression in slow- and fast-twitch skeletal muscle in parallel with improvements in insulin-stimulated glucose uptake. HSP72 induction was also observed in epididymal adipose tissue and liver (154). Our findings and others demonstrate that *in vivo* heat treatments and transgenic overexpression of skeletal muscle HSP72 reduced epididymal fat pad mass in rodents fed a high-fat diet (67, 154), and heat treatment reduced plasma triglycerides and free fatty acids in *db/db* mice (256). Drew *et al.* recently expanded upon these observations by showing that global knock-out of HSP72 induced an insulin resistant phenotype with increased adipose tissue mass (101). Despite the observed induction of HSPs in epididymal adipose tissue, and the reduction in free fatty acids and adipose tissue mass with *in vivo* heat treatments, very little is known about the expression patterns and function of HSPs in adipose tissue.

HSPs are molecular chaperones that aid in protein refolding and prevent protein aggregation (135, 354). HSP72 is a cytosolic chaperone, is highly inducible following heat stress, and has anti-inflammatory and anti-apoptotic properties (135, 354). HSP25 is another cytosolic chaperone that plays a role in cytoskeletal dynamics and also has anti-inflammatory functions (135, 354). HSP60 is largely a mitochondrial chaperone shown to be essential for proper folding of imported mitochondrial proteins (95, 354). HSP expression in skeletal muscle corresponds with oxidative capacity with higher constitutive, or basal/unstressed levels, of HSP expression observed in slow-twitch oxidative skeletal muscle compared to fast-twitch glycolytic

skeletal muscle (45, 278, 296, 361). As a result, muscle fiber type and constitutive HSP expression levels play an important role in determining HSP function in skeletal muscle (154).

There are significant metabolic differences between WAT present in the subcutaneous depots and WAT present in the abdominal cavity (retroperitoneal and epididymal depots) (139, 207). Adipocytes from abdominal depots have greater secretion of inflammatory cytokines (108, 207, 385, 505), greater lipolysis and fatty acid re-esterification (14, 170, 277, 481), and greater oxygen consumption and mitochondrial protein content (96, 264). It is currently unknown as to whether HSP expression corresponds with metabolic activity in adipose tissue depots.

The purpose of the present study was two-fold: First, to determine if HSP expression varied across WAT depots (subcutaneous, epididymal, and retroperitoneal); and second, to determine if the induction of HSPs following a single heat treatment was WAT depot-specific. We also examined the impact of an acute bout of heat treatment on adipose tissue lipolysis and insulin responsiveness. We hypothesized that HSP expression patterns would parallel the metabolic activity of the WAT depots, with greater HSP expression demonstrated in more metabolically active depots (i.e., retroperitoneal and epididymal depots) (139, 207). Additionally, we hypothesize that HSP induction would be greatest in WAT depots where constitutive HSP levels were low. These differences between WAT depots could have potential implications for HSP function in adipose tissue health and disease.

2.3. MATERIALS AND METHODS

Materials. HSP72 primary antibody (Cat # SPA-810) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Phospho-HSP25 (Ser-82) (Cat # 905-642), total HSP25 (Cat # SPA-801), and HSP60 (Cat # SPA-807) primary antibodies were purchased from Stressgen (Victoria, British Columbia, Canada). Phospho-AMPK (Thr-172) (Cat # 2535), total AMPK (Cat # 2603), phospho-HSL (Ser-660) (Cat # 4126), total HSL (Cat # 4107), phospho-Akt (Ser-473) (Cat # 9271), total Akt (Cat # 9272), phospho-AS-160 (Thr-642) (Cat # 4288), and ATGL (Cat # 2138) primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). PEPCK primary antibody (Cat # 100004943) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). PDK4 primary antibody (Cat # ab89295) and horseradish peroxidase (HRP)-conjugated β -actin primary antibody (Cat # ab20272) were purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit HRP-conjugated secondary antibody (Cat # sc-2004) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Donkey anti-rabbit HRP-conjugated secondary antibody (Cat # 711-035-152) was purchased from Jackson Immuno-Research, Inc. (West Grove, PA, USA). Goat anti-mouse HRP-conjugated secondary antibody (Cat # 170-5047) and Bradford protein quantification reagent were purchased from Bio-Rad (Hercules, CA, USA). NEFA standard and reagents were purchased from Wako Chemicals (Dallas, TX, USA). Insulin and leptin ELISA kits were purchased from Alpco Diagnostics (Salem, NH, USA). Cell extraction buffer was purchased from Invitrogen (Camarillo, CA, USA). Enhanced chemiluminescence reagents were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Insulin was purchased from Novo Nordisk (Princeton, NJ, USA). Free glycerol standard and reagent, epinephrine, and all other reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise specified.

Experimental animals & in vivo heat treatment. Fourteen week old male Wistar rats (~350 – 375 g each) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a temperature controlled facility ($22 \pm 2^{\circ}\text{C}$) with 12:12 hr light:dark cycles. Animals were allowed *ad libitum* access to water and a standard chow diet (9604; Harlan Teklad, Madison WI, USA) and divided into sham- (n = 6) or heat-treated (n = 5) groups. All animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) prior to treatment. Heat treatment consisted of lower body immersion in a 42°C water bath to gradually increase body temperature to between 41°C and 41.5°C where it was maintained for 20 min. Sham treatment consisted of immersion in a 37°C water bath and maintaining body temperature at 37°C for 20 min (154). Body temperature was monitored by a rectal thermometer. In earlier preliminary experiments using male Wistar rats of similar age, size, and chow diet, the time to reach 41°C rectal temperature ranged from 18 – 24 min. In total, the rats were exposed to heat treatment for less than 45 min, a duration that would not typically be considered equivalent to heat stroke. Following treatment, 0.5 ml of 0.9% saline were injected intraperitoneally to aid in recovery. Twenty-four hours following heat or sham treatment, and following a 10-hr overnight fast, animals were again anesthetized with pentobarbital sodium and tissues dissected for experimental procedures. All protocols and procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Blood measures. Blood was drawn from the chest cavity during dissection and blood glucose concentration determined immediately using a glucose analyzer and the manufacturer's test strips (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN, USA). Blood was then allowed to clot for 30 min on ice, spun at 3,000 g for 60 min at 4°C , and serum drawn off and frozen at -80°C . Serum was analyzed for insulin and leptin concentration by ELISA, and for

concentration of non-esterified free fatty acids (FFA) and free glycerol using colorimetric assays according to the manufacturer's instructions.

Ex vivo adipose tissue explants following in vivo sham or heat treatment. Adipose tissue explants were used to assess whether a single *in vivo* heat treatment altered adipose tissue metabolism by examining basal- and epinephrine (Epi)-stimulated lipolysis and fatty acid re-esterification, as well as responsiveness to insulin (512). In brief, inguinal SCAT, eWAT, and rpWAT WAT depots were removed from male Wistar rats 24 hr following a single *in vivo* sham or heat treatment. Adipose was dissected into ~100 mg explants and placed in vials in a 37°C water bath containing 3 ml of oxygenated Krebs-Ringer buffer (118 mmol/l NaCl, 4.8 mmol/l KCl, 1.25 mmol/l CaCl₂, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 25 mmol/l NaHCO₃, and 5 mmol/l glucose; pH=7.4) supplemented with 2.5% FA-free BSA. Temperature was maintained at 37°C throughout the experiment. Explants were allowed to recover for 15 min after which 100 µl of media was removed and stored at -20°C. Following recovery, media was supplemented with either water (basal), Epi (10 µmol/l), or insulin (100 µU/ml) for 2 hr after which explants and media were separated using a cell strainer. Media was frozen and stored at -20°C. Tissue was frozen in liquid nitrogen and stored at -80°C. Insulin incubated explants were saved for Western blotting only. Media was assayed for the concentration of free glycerol and FFA to assess basal and Epi-stimulated lipolysis. Free glycerol and FFA release were normalized to mg tissue weight for comparisons between sham and heat treated animals and were normalized to µg protein content measured by Bradford assay performed in triplicate to compare WAT depots. Epi-stimulation increased glycerol and FFA release in all adipose tissue depots examined and was used as a positive control; however, Epi responsiveness was not changed following a single *in vivo* heat treatment (data not shown). Fatty acid re-esterification was assessed by quantifying the

ratio of FFA to free glycerol (FFA/glycerol) where a ratio near 3.0 represents an absence of fatty acid re-esterification, and a ratio near 0.0 represents complete re-esterification of fatty acids into triglycerides (277, 512). Absolute rates of fatty acid re-esterification were calculated as the differences between 3 times glycerol (theoretical fatty acid release) and the measured fatty acid release (500, 511). This calculation assumes negligible fatty acid oxidation.

Adipose tissue organ culture. In a separate set of experiments, SCAT, eWAT, and rpWAT were removed and cultured as previously described (124, 468, 511, 512) from approximately 14-wk old male Wistar rats weighing ~350 – 375 g each (n = 6). Under sterile conditions, adipose tissue from each depot was minced and placed into separate culture dishes (approximately 500 mg of adipose tissue per dish), one dish designated for heat treatment per depot and one dish designated for sham treatment per depot. This was repeated for each of the six animals: sham treatment (n = 6) and heat treatment (n = 6) for each adipose tissue depot. Culture dishes contained 15 ml of M-199 supplemented with 1% antibiotic-penicillin-streptomycin, 50 μ U/ml insulin, and 2.5 nmol/l dexamethasone. Culture dishes were placed in an incubator overnight maintained at 37°C with a gas phase of 5% CO₂. After a 24-hr recovery incubation, adipose tissue cultures from each depot were placed either in a 42°C water bath for 30 min or maintained at 37°C. After heat treatment, cultures were placed back into the incubator and 24-hr later culture medium containing the adipose tissue was poured into ice-cold PBS, filtered, and remaining adipose tissue samples were frozen at -80°C.

Western blotting. WAT from *in vivo* sham- and heat-treated animals not used for *ex vivo* adipose tissue explants, as well as minces from ATOC were frozen in liquid nitrogen and stored at -80°C to be processed for Western blotting by methods previously described (154, 155). WAT

was homogenized in a 2:1 (volume-to-weight) ratio of ice-cold cell extraction buffer containing 10 mmol/l Tris·HCl (pH 7.4); 100 mmol/l NaCl; 1 mmol/l each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mmol/l Na_3VO_4 ; 20 mmol/l $\text{Na}_4\text{P}_2\text{O}_7$; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250 μl /5 ml protease inhibitor cocktail.

Homogenates were sonicated for 3 – 5 s, rotated for 30 min at 4°C, and then centrifuged for 20 min at 3,000 rpm at 4°C. The infranatant was removed and protein concentration determined by Bradford assay performed in triplicate. Samples were diluted in HES buffer (20 mmol/l HEPES, 1 mmol/l EDTA, 200 mol/l sucrose, pH 7.4) and 5 x Laemmli buffer containing 100 mmol/l dithiothreitol (Thermo Scientific, MA, USA) based on protein concentration to generate samples containing the same concentration of protein for analysis by SDS-PAGE. Samples were heated in a boiling water bath for 5 min.

Protein (10 – 30 μg) was separated on 7.5% – 10% SDS-PAGE gels, followed by a wet transfer to a nitrocellulose membrane for 60 – 90 min at 200 mA. Membranes were Ponceau stained to verify even protein loading. Membranes were blocked for 1 hr at room temperature in Tris-buffered saline, 0.1% Tween 20 (TBST), and 5% nonfat dry milk or 1% BSA, followed by an overnight incubation with the appropriate primary antibodies. Primary antibodies were diluted in TBST with 1% nonfat dry milk or 1% – 5% BSA at a concentration of 1:500 or 1:1,000. Following three brief washes with TBST, blots were incubated for 1 hr at room temperature in TBST 1% nonfat dry milk supplemented with an appropriate HRP-conjugated secondary antibody at a concentration of 1:5,000 or 1:10,000. Blots were then washed twice with TBST and once with TBS. Blots were visualized by Enhanced chemiluminescence (ECL). Bands were quantified using Image J densitometry. Blots for *in vivo* experiments were then stripped and re-probed for β -actin as a loading control. Blots for *in vitro* experiments were normalized to

Ponceau staining. Blots were stripped for 15 – 20 min at 55°C in buffer containing 62.5 mmol/l Tris·HCl, 2% SDS, and 100 mmol/l 2-mercaptoethanol.

Statistical analyses. Results are presented as mean \pm SEM. Statistical significance was set at $P < 0.05$ and analyses performed using Sigma Plot for Windows, version 12.0 (Systat Software Inc., Chicago, IL, USA). One-way ANOVAs were performed to test for significant differences between treatment groups and between adipose tissue depots. Tukey's post hoc comparisons were performed when necessary. A paired t-test was performed to test for significant differences between basal free glycerol release, FFA release, and FFA/glycerol ratio, between basal- and insulin-stimulated protein phosphorylation, and between ATOC treatment groups. Linear regression was performed to determine correlations between constitutive, unstressed HSP levels and basal glycerol release from *ex vivo* WAT explants, as well as FFA/glycerol. Where raw values did not meet the ANOVA assumptions of normally distributed data or equal variance, the raw values were transformed logarithmically or by square root to meet these assumptions. Raw values are presented in all figures.

2.4. RESULTS

Basal HSP expression. We observed that HSP72 levels were greatest in eWAT, intermediate in rpWAT, and lowest in SCAT from sham treated animals (eWAT and rpWAT: ~5.4-fold and ~2.9-fold greater than SCAT, respectively, and eWAT: ~1.9-fold greater than rpWAT) (**Fig. 3A**). Total HSP25 levels were ~5.8-fold greater and ~6.0-fold greater in the eWAT and rpWAT compared to SCAT, respectively (**Fig. 3B**). There were no differences in

phospho-HSP25 (Ser-82) relative to HSP25 and in HSP60 between WAT depots (**Figs. 3C & 3D**).

Induction of HSPs by heat treatment. HSP72 content was significantly increased in all three depots 24 hr following *in vivo* heat treatment, and the induction of HSP72 was greatest in the SCAT (~6.8-fold increase) (**Fig. 3A**). Total HSP25 levels were increased in the eWAT and rpWAT, but not different in SCAT (**Fig. 3B**). There were no changes in phospho-HSP25 relative to total HSP25 following heat treatment (**Fig. 3C**). HSP60 levels were increased following heat treatment in the rpWAT, but unchanged in the other depots (**Fig. 3D**).

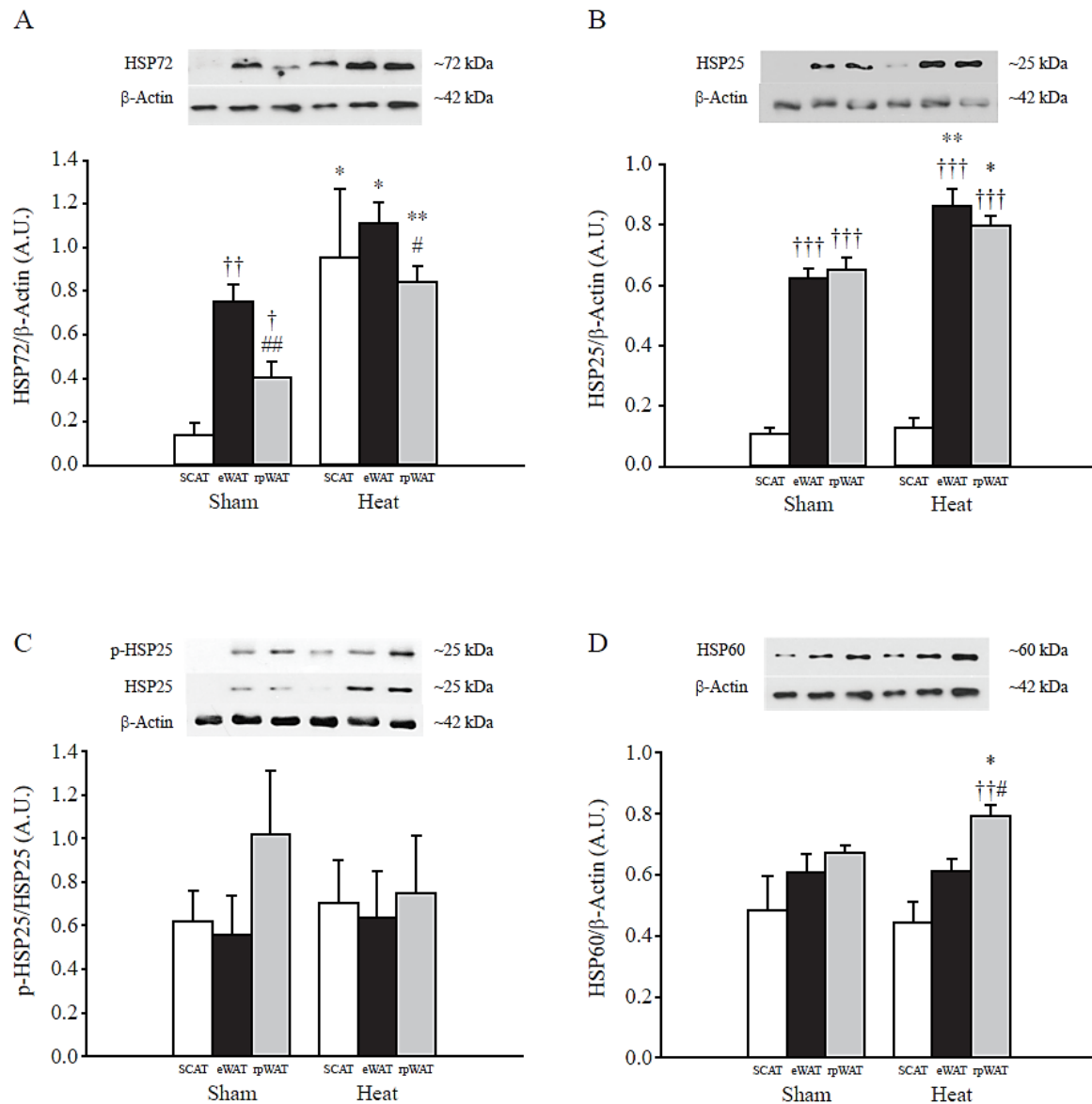


Figure 3. HSP expression and induction following a single heat treatment *in vivo*.

HSP72 (A), HSP25 (B), phospho-HSP25 (C), and HSP60 (D) levels between WAT from SCAT, eWAT, and rpWAT depots. Male Wistar rats received a single heat treatment (41°C for 20 min) or sham treatment (37°C for 20 min). Twenty-four hours following heat or sham treatment, WAT depots were removed and protein expression measured by Western blot. Protein levels were normalized to β -actin protein levels. * $P < 0.05$, ** $P < 0.01$ denotes a significant increase following heat treatment determined by ANOVA. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ denotes that eWAT or rpWAT are significantly different from SCAT determined by ANOVA. # $P < 0.05$, ## $P < 0.01$ denotes that eWAT is significantly different from rpWAT determined by ANOVA. Values are means \pm S.E.M. $N = 3$ -6 samples per group in SCAT, $N = 5$ -6 samples per group in eWAT and rpWAT.

To determine if the observed differences in the induction of HSP expression across WAT depots were influenced by variable distribution of heat *in vivo*, we performed heat treatment on adipose tissue organ cultures. We removed adipose tissue from a subset of control rats, placed the depots in culture, and then exposed each depot to heat treatment *in vitro*. Similar to what was observed *in vivo*, HSP72 increased following heat treatment in all three depots with the greatest induction in HSP72 occurring in the SCAT (**Fig. 4A**). HSP25 expression significantly increased in all three depots (**Fig. 4B**) while phospho-HSP25 relative to total HSP25 was unchanged (**Fig. 4C**). HSP60 significantly increased following heat treatment in the SCAT and rpWAT, while *in vivo* only the rpWAT showed an induction in HSP60 with heat treatment (**Fig. 4D**). *In vitro*, the basal expression of HSPs was not significantly different between depots. Overall, the induction of HSP72 is most robust in SCAT both *in vivo* and *in vitro*, and the HSP induction patterns in rpWAT and eWAT were consistent between *in vivo* and *in vitro* experiments. Interestingly, the SCAT was more responsive to *in vitro* heat treatment with significant increases in both HSP25 and HSP60, a pattern not observed *in vivo*.

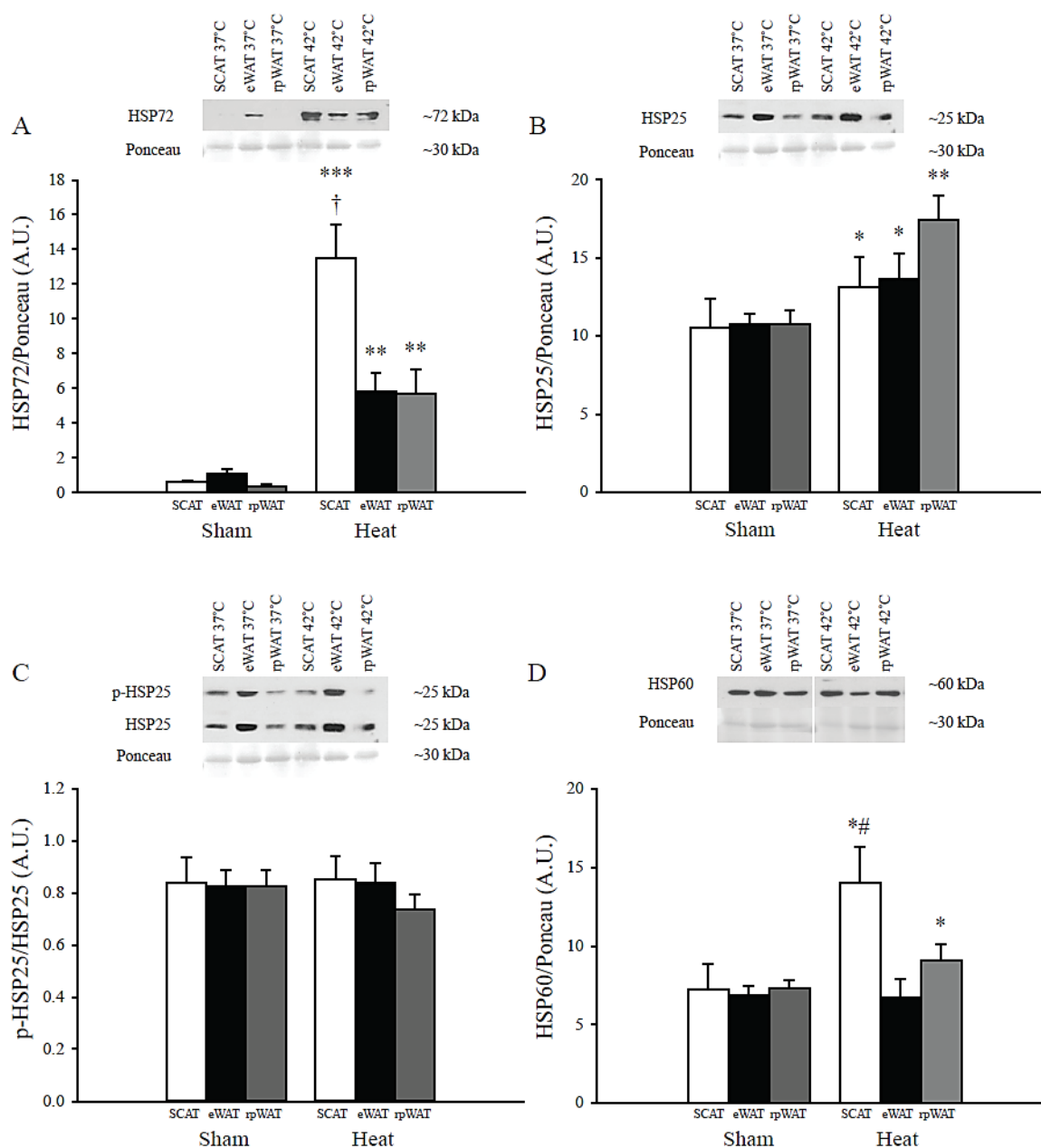


Figure 4. Induction of HSPs in response to *in vitro* heat treatment

HSP72 (A), HSP25 (B), phospho-HSP25 (Ser-82) (C), and HSP60 (D) expression in white ATOCs following a single *in vitro* heat treatment. Adipose tissue from SCAT, eWAT, and rpWAT, were removed from anesthetized male Wistar rats, minced, and placed in separate culture dishes. HSP expression was determined by Western blotting and protein levels normalized to Ponceau staining. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denotes a significant increase following heat treatment determined by paired t-test. † $P < 0.05$ denotes that the induction of HSP expression is significantly greater in the SCAT compared to the eWAT and rpWAT. # $P < 0.05$ denotes that the induction of HSP expression is significantly greater in the SCAT compared to eWAT only determined by ANOVA. Values are mean fold change \pm S.E.M. $N = 5-6$ samples per group.

The impact of heat treatment on lipolysis and fatty acid re-esterification. Twenty-four hours after a single *in vivo* heat treatment, circulating glucose, insulin, glycerol, NEFA, or leptin concentrations were not significantly altered (**Table 1**).

Table 1: Blood measures following a single *in vivo* heat treatment.

	Sham treated	Heat treated	<i>P</i> -value
Glucose (mg/dl)	137.0 ± 3.7	133.2 ± 4.6	<i>P</i> = 0.53
Insulin (ng/ml)	1.3 ± 0.3	1.3 ± 0.3	<i>P</i> = 0.96
Leptin (ng/ml)	0.53 ± 0.13	0.57 ± 0.13	<i>P</i> = 0.82
Glycerol (mmol/l)	0.17 ± 0.04	0.22 ± 0.03	<i>P</i> = 0.44
NEFA (mmol/l)	0.37 ± 0.06	0.45 ± 0.05	<i>P</i> = 0.31

Values are means ± S.E.M. *N* = 5-6 samples per group.

When examining markers of lipolysis in individual depots, basal free glycerol release from *ex vivo* adipose tissue explants was greatest in eWAT, intermediate in rpWAT, and lowest in SCAT when expressed relative to total protein content (eWAT: 0.054 ± 0.002 $\mu\text{mol}/\mu\text{g}$ protein/2 hr, rpWAT: 0.042 ± 0.003 $\mu\text{mol}/\mu\text{g}$ protein/2 hr, SCAT: 0.014 ± 0.001 $\mu\text{mol}/\mu\text{g}$ protein/2 hr, *P* < 0.01). In addition, basal glycerol release was significantly correlated with constitutive HSP72 (**Fig. 5A**) and HSP25 levels from sham treated animals (**Fig. 5B**). There were no differences in FFA release across adipose depots (data not shown). Paralleling lipolysis, basal FFA/glycerol was lowest in eWAT, intermediate in rpWAT, and greatest in SCAT from sham treated animals (**Figs. 6A – 6C**), indicative of greater fatty acid re-esterification in eWAT, intermediate in rpWAT, and lowest in SCAT. We also observed that basal FFA/glycerol negatively correlated with constitutive HSP72 (**Fig. 5C**) and HSP25 levels (**Fig. 5D**), indicating that constitutive HSP72 and HSP25 levels in WAT were positively correlated with fatty acid re-esterification.

Following a single heat treatment, fatty acid re-esterification was changed in a depot specific fashion. Basal FFA/glycerol in rpWAT was significantly increased following heat treatment, indicative of a decrease in fatty acid re-esterification (**Fig. 6A**). Conversely, basal FFA/glycerol in SCAT was significantly reduced following heat treatment (**Fig. 6C**). There were no differences in fatty acid re-esterification in eWAT following heat treatment (**Fig. 6B**). Absolute rates of fatty acid re-esterification paralleled the results indicated by FFA/glycerol; however, were not significantly different between sham and heat treatments (**Table 2**). Following heat treatment, there were no changes in glycerol release in any of the adipose tissue depots examined. FFA release was increased following heat treatment in the rpWAT, but was not altered in eWAT or SCAT (data not shown).

Table 2. Absolute Rates of Basal Fatty Acid Re-Esterification

WAT depot	Sham treated	Heat treated	<i>P</i>-value
rpWAT (μmol/g tissue/2 hr)	7.10 ± 0.86	6.16 ± 0.45	<i>P</i> = 0.358
eWAT (μmol/g tissue/2 hr)	11.96 ± 1.30	12.35 ± 1.17	<i>P</i> = 0.826
SCAT (μmol/g tissue/2 hr)	1.52 ± 0.60	3.10 ± 0.73	<i>P</i> = 0.134

Values are means ± S.E.M. *N* = 5-6 samples per group.

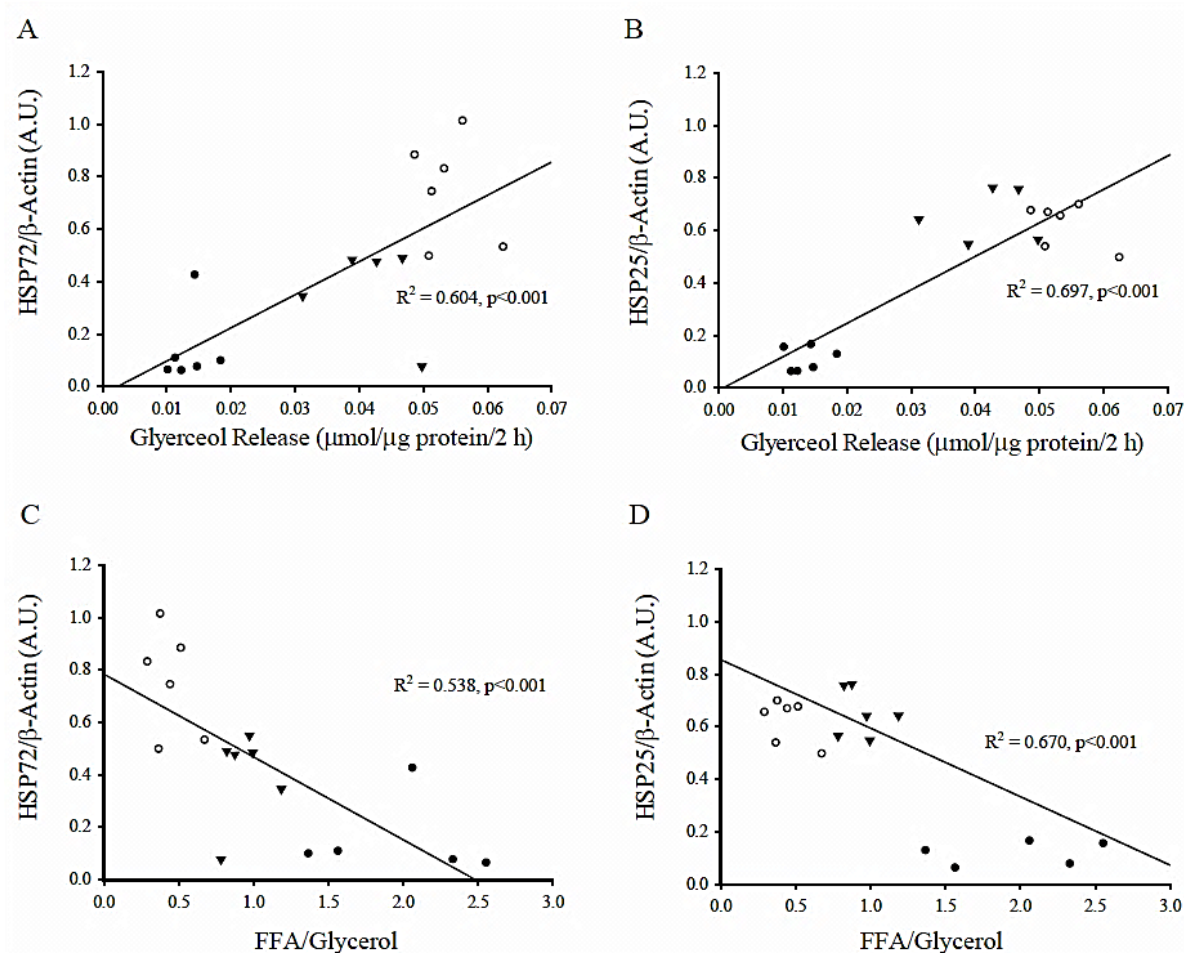


Figure 5. Correlations between HSP72, HSP25, lipolysis and fatty acid re-esterification. Correlations between basal HSP72 (A & C) and HSP25 (B & D) expression levels from SCAT, eWAT, and rpWAT and basal free glycerol release (A-B) and basal FFA/glycerol (C-D) from *ex vivo* adipose tissue explants. Twenty-four hours following *in vivo* sham treatment, WAT depots were removed and protein expression measured by Western blot. Protein levels were normalized to β-actin protein levels. WAT from each depot was also used for *ex vivo* adipose tissue explants, and free glycerol release into the media were assayed using colorimetric assays and normalized to protein content (μmol/μg protein/2 hr). Basal fatty acid re-esterification was quantified as the ratio of glycerol release to FFA release (FFA/glycerol). ● represent basal SCAT samples, ▲ represent basal rpWAT samples, and ○ represent basal eWAT samples.

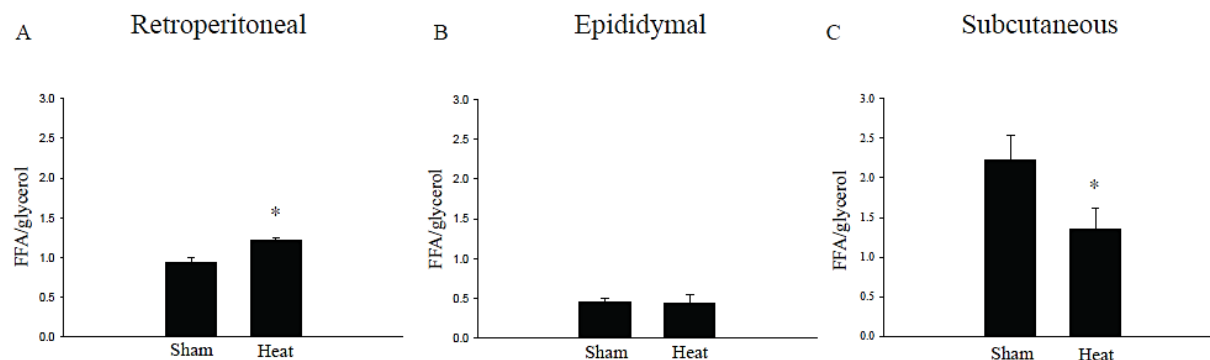


Figure 6. Fatty acid re-esterification from *ex vivo* adipose tissue explants following heat treatment

Fatty acid re-esterification (A-C) from *ex vivo* adipose tissue explants removed from male Wistar rats 24 hr following a single *in vivo* heat or sham treatment. *Ex vivo* adipose tissue explants from the rpWAT, eWAT, and SCAT depots were removed for *ex vivo* assessment of free glycerol and FFA release in order to determine fatty acid re-esterification. Fatty acid re-esterification was quantified as the ratio of FFA release to glycerol release (FFA/glycerol). * $P < 0.05$ denotes that heat treatment was significantly different than sham treatment in untreated, basal explants determined by ANOVA. Values are means \pm S.E.M. $N = 5-6$ samples per group.

We examined protein levels and phosphorylation of a number of lipolytic enzymes and enzymes involved in fatty acid re-esterification, but did not observe differences between phosphorylated hormone sensitive lipase (HSL) (Ser-660), total HSL, total adipose triglyceride lipase (ATGL), phosphoenolpyruvate carboxylase (PEPCK), and pyruvate dehydrogenase 4 (PDK4) between sham and heat treated animals in any of the WAT depots examined.

Insulin responsiveness following in vivo heat treatment. *Ex vivo* adipose tissue explants from the rpWAT, eWAT, and SCAT removed 24 hr following either *in vivo* heat or sham treatment were incubated with insulin (100 μ U/ml) to determine insulin responsiveness. Phosphorylation of Akt (Ser-473) was significantly increased in the rpWAT and eWAT following insulin incubation, but was not significantly increased in SCAT (**Figs. 7A – 7C**). Phosphorylation of AS-160 (Thr-642) was also significantly increased following insulin incubation in the rpWAT and eWAT, but not significantly increased in SCAT (**Figs. 7D – 7F**). Furthermore, we observed that phosphorylation of Akt (**Fig. 7B**) and AS-160 (**Fig. 7E**) following insulin incubation was significantly greater in eWAT explants from heat treated animals compared to eWAT explants from sham treated animals, but was not significantly different in rpWAT (**Figs. 7A & 7D**) or SCAT (**Figs. 7C & 7F**).

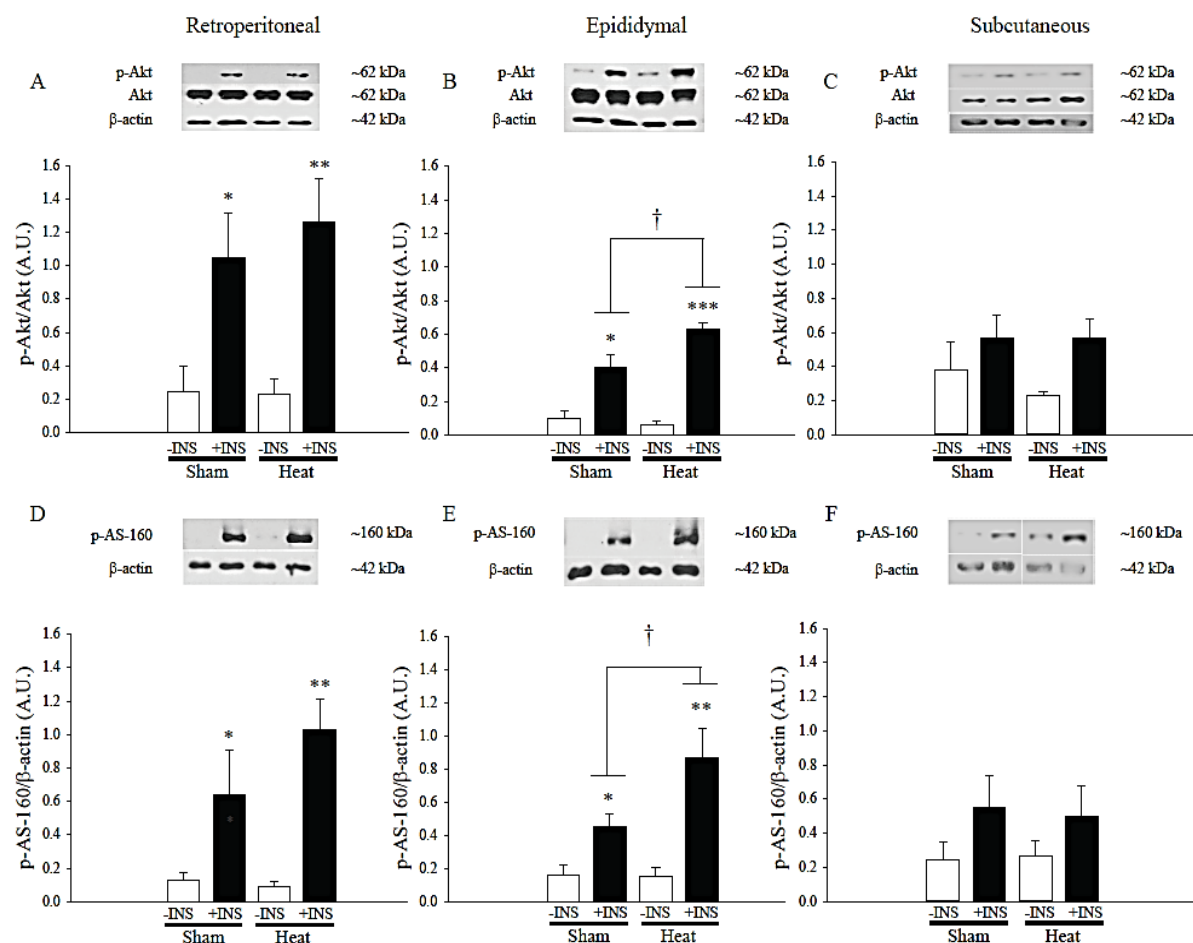


Figure 7. Insulin response following a single *in vivo* heat treatment.

Phosphorylation of Akt and AS-160 in explants from the retroperitoneal (A & D), epididymal (B & E), and subcutaneous (C & F) WAT depots. Adipose tissue explants were removed from male Wistar rats 24 hr following a single *in vivo* heat or sham treatment for *ex vivo* assessment of insulin responsiveness. Explants were incubated with water (-INS) or insulin (+INS) (100 μ U/ml) for 2 hr after which explant tissue was removed and frozen at -80°C . Phosphorylation of Akt (Ser-473), total Akt, and phosphorylation of AS-160 (Thr-642) were determined by Western blotting. Phospho-Akt protein levels were normalized total Akt protein levels, and phospho-AS-160 protein levels were normalized to β -actin protein levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denotes that insulin stimulated condition was greater than non-insulin stimulated condition determined by paired t-test. † $P < 0.05$ denotes that insulin stimulated condition in adipose tissue explants from heat treated animals was significantly greater than insulin stimulated condition in explants from sham treated animals determined by ANOVA. Values are means \pm S.E.M. $N = 5-6$ samples per group.

2.5. DISCUSSION

New data from the present study demonstrate depot-specific patterns of HSP expression with higher HSP72 and HSP25 expression in more metabolically active WAT depots (i.e., eWAT and rpWAT). In addition, an acute *in vivo* heat treatment induces a unique HSP response across WAT depots. In general, HSP72 and HSP25 are highly inducible in WAT depots, with the greatest induction of HSP72 occurring in the SCAT – a depot with the lowest constitutive expression of this protein. These results are for the most part recapitulated when examined *in vitro*. While an association between HSP expression and oxidative capacity has been established in skeletal muscle, the relationship between HSP expression and adipose tissue metabolism is less clear. Variability in HSP expression and induction patterns further supports the growing body of literature delineating the function of various WAT depots in health and disease.

HSP expression in the adipose tissue. In the present study, we observed marked differences in the expression levels and induction of HSPs in response to heat treatment across adipose tissue depots. Constitutive HSP72 levels demonstrated the pattern of eWAT > rpWAT > SCAT, while HSP25 was similar with eWAT/rpWAT > SCAT. Differences in HSP72 and HSP25 expression across adipose tissue depots observed in the present study may involve differences in the regulation or expression of HSF-1, the primary transcriptional regulator of HSP72 and HSP25 (329, 354). The differences observed in HSP expression between adipose tissue depots are in agreement with other laboratories that have reported greater HSP content in more metabolically active WAT depots of mice and humans (379, 383, 421). Perez-Perez *et al.* reported that in humans HSP72, HSP25, and HSP90 expression levels were higher in omental adipose tissue compared to SCAT, in support of our findings (383). In another human study,

Peinado *et al.* did not find differences in HSP72 expression between SCAT and visceral adipose tissue, or differences between adipocytes isolated from these depots. These investigators also found significantly greater HSP72 expression in the SVF of SCAT compared to SVF of visceral adipose tissue (379). There are differences in adipose tissue depots between humans and rodents and this could account for the observed differences between our study and Peinado *et al.* (379). In addition, Peinado *et al.* examined lean, but older subjects (50 – 70 years of age) and both HSP expression and induction of the heat shock response have been shown to be decreased with age (152, 443).

Our findings clearly demonstrate that HSP expression patterns differ across adipose tissue depots, and that the response to heat treatment is depot-specific as well. Induction of HSP72 and HSP25 was robust in all depots both *in vivo* and *in vitro*, with the exception of SCAT where induction of HSP25 did not occur *in vivo*. Typically, HSP72 and HSP25 are inducible to heat and more directly involved in the tissue stress response, in comparison to HSP60, which is primarily a mitochondrial chaperone protein (95, 354). This is supported by our findings where induction of HSP60 occurred only in the rpWAT *in vivo*. Changes in phosphorylation of HSP25 were not observed either *in vivo* or *in vitro* in any of the adipose tissue depots examined, despite significant induction of total HSP25 protein with heat treatment. It could be that changes in phosphorylation of HSP25 are not evident at the 24 hr time point utilized in the present study. Differences between adipose tissue depots in the sensitivity and response to temperature have been observed in other experimental situations. Recently the SCAT of mice has been shown to be more sensitive to cold exposure (10°C for 20 h) than eWAT, as measured by the induction of peroxisome proliferator-activated receptor gamma co-activator-alpha (PGC-1 α) and uncoupling protein-1 (UCP-1) mRNA (535). Future studies are needed to determine the effects of age and

obesity on HSP expression and induction, as well as identify which cells in the mixed cell population of adipose tissue underlie observed HSP expression patterns.

Effects of heat treatment on WAT metabolism. The effect of *in vivo* heat treatment on WAT metabolism has previously not been well described. Early work by Torlinska *et al.* pointed towards a reduction in lipolysis shortly following heat treatment in rats (482). In the present study, we observed distinct changes in WAT metabolism between depots 24 hr following a single heat treatment. We observed the most pronounced changes in the rpWAT where basal lipolysis was increased and fatty acid re-esterification was decreased. In the SCAT, we observed an increase in basal fatty acid re-esterification without changes in lipolysis. As a result of obesity and metabolic dysfunction, the SCAT loses some of its ability to store fatty acids. This results in fatty acid storage in abdominal WAT depots and organs (i.e., skeletal muscle) and further metabolic dysfunction (415). The increase in fatty acid re-esterification observed here in the SCAT following heat treatment may be of metabolic benefit during pro-obesity and pro-inflammatory conditions to help promote proper fatty acid storage. In essence, the robust increase in HSP72 observed in this depot may result in anti-inflammatory effects which protect against obesity-induced adipose dysfunction. Furthermore, following heat stress, HSP72 localizes to the lipid droplet surface in isolated adipocytes (223) and heat stress has been shown to modulate lipid membrane integrity (483, 484, 490). These processes may help explain the observed changes in lipolysis and fatty acid re-esterification without observed changes in enzyme levels. We also observed that following a single *in vivo* heat treatment, insulin responsiveness increased in eWAT, but this enhanced insulin responsiveness did not occur in rpWAT and SCAT. This new insulin data further highlights the depot-specific response to heat treatment. Together, the short-term changes in fatty acid metabolism observed following a single

heat treatment may result in improved metabolic profile, an idea that could lead to new understanding of the adipose tissue stress response.

CONCLUSION

The results of the present study indicate that HSP levels are greater in more metabolically active WAT depots. The induction of HSP72 occurs in all WAT depots examined and is greatest following a single heat treatment in the SCAT. The induction of HSP25 occurs in the rpWAT and eWAT *in vivo* and in all WAT depots *in vitro*. A single heat treatment alters WAT lipolysis, fatty acid re-esterification, and insulin responsiveness in a depot specific fashion. Future studies are needed to determine the effects of chronic heat treatment and HSP induction on adipose tissue function and potential protection from diet-induced obesity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

CHAPTER 3

WEEKLY HEAT TREATMENTS IMPROVE THE METABOLIC PROFILE OF MULTIPLE METABOLIC TISSUES IN RATS FED A HIGH-FAT DIET

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Manuscript in Preparation

3.1. ABSTRACT

We have observed that weekly *in vivo* heat treatments in conjunction with high-fat feeding prevent the development of glucose intolerance and skeletal muscle insulin resistance. The purpose of the current study was to determine whether weekly *in vivo* heat treatment can improve glucose intolerance and skeletal muscle insulin sensitivity in rats fed a high-fat diet (HFD), as well as determine the effects of heat treatment on other metabolic tissues. Male Wistar rats were fed a HFD consisting of 60% of kilocalories from fat for 8 wk. After 8 wk, the HFD was continued and animals received weekly *in vivo* sham (37°C, 20 min) or heat treatment (41°C) for 7 wk. After 6 wk, animals underwent an intraperitoneal glucose tolerance test. 24-48 hr following the final sham or heat treatment, skeletal muscle was harvested and processed for insulin-stimulated glucose uptake and Western blotting. The epididymal adipose tissue and liver were also removed. Heat treatment reduced glucose intolerance while increasing insulin-stimulated glucose uptake in the EDL and reducing the triglyceride content of another glycolytic muscle, the tibialis anterior, similar to the EDL. HSP72 and HSP60 levels increased in the EDL following heat treatment, but did not change in the soleus. PGC-1 α was reduced by heat treatment in the EDL ($P = 0.08$) and the soleus ($P < 0.05$). In the liver, triglyceride content was reduced by heat treatment and HSP72 and HSP25 levels were increased along with levels of microtubule-associated protein 1 light chain 3 (LC3-II). In the white adipose tissue (WAT), adipocyte size was reduced by heat treatment and expression of HSP72 and PGC-1 α was increased. Weekly heat treatment can improve metabolic profile of several tissues in established insulin resistance. Treatments that induce expression of HSP72 should be explored as treatment options for metabolic conditions, including non-alcoholic fatty liver disease.

Key words:

Heat shock proteins, insulin resistance, type 2 diabetes, skeletal muscle, non-alcoholic fatty liver disease (NAFLD).

3.2. INTRODUCTION

HSPs and the heat shock response are highly conserved mechanisms by which cells and organisms guard against heat and other stressors (328, 329). HSP72 is of particular interest due to its highly inducible nature in response to stressors including endurance exercise in both the skeletal muscle and liver (142, 265, 266, 329, 334). It was discovered more than a decade ago that HSP72 expression is lower in the skeletal muscle of type 2 diabetics (269, 375), which has since been observed by other investigators (50, 67). Recently, HSP72 levels have been found to be negatively associated with the progression of hepatic steatosis and insulin resistance in the liver (97).

We and others have shown that induction of HSP72 by genetic modification, *in vivo* heat treatments, or pharmacological means can improve the metabolic profile of insulin resistant rodents (3, 28, 67, 154, 173, 256), while the loss of HSP72 results in whole-body and skeletal muscle insulin resistance (101). HSP72 has been shown to regulate skeletal muscle mitochondrial dynamics and mitophagy – the autophagic process by which damaged mitochondria are degraded (99, 101, 173). Loss of HSP72 results in retention of dysmorphic mitochondria, but transgenic overexpression of skeletal muscle HSP72 results in an increase in mitochondrial content and activity of mitochondrial enzymes (101, 173). The positive role that HSP72 induction and heat treatment may have on the liver has not been widely studied. The induction of HSP72 in the liver may be a viable treatment option for reducing lipid storage and inflammation.

The purpose of the present study was two-fold. First, to determine whether *in vivo* heat treatments that induce HSP72 expression would improve insulin sensitivity in rats following 8

wk of high-fat feeding. Previously we had noted that 12 wk of weekly heat treatments in conjunction with 12 wk high-fat feeding protected against the development of insulin resistance (154). A second purpose was to determine whether *in vivo* heat treatment would result in metabolic improvements in the liver and adipose tissue as has previously been shown in skeletal muscle.

3.3. METHODS

Experimental animals & in vivo heat treatment. Eight wk old male Wistar rats (~150 – 180 g each) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a temperature controlled facility ($22 \pm 2^\circ\text{C}$) with 12:12 hr light:dark cycles. Animals were allowed *ad libitum* access to water. Rats were fed a modified Kraegen HFD (60% of kilocalories from fat) for 15 wk (154, 462). During the last 7 wk of the HFD, rats received either weekly *in vivo* heat or sham treatment (n=9/group). All animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) prior to sham or heat treatment. Heat treatment consisted of lower body immersion in a 42°C water bath to gradually increase body temperature to between 41°C and 41.5°C where it was maintained for 20 min. Sham treatment consisted of immersion in a 37°C water bath and maintaining body temperature at 37°C for 20 min (154, 412). Body temperature was monitored by a rectal thermometer. Following treatment, 0.5 ml of 0.9% saline were injected intraperitoneally to aid in recovery. Forty-eight hr following heat or sham treatment, and following a 10-hr overnight fast, animals were again anesthetized with pentobarbital sodium and tissues dissected for experimental

procedures. All protocols and procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Glucose tolerance testing and other blood measures. One wk prior to sacrifice, and 48 hr following heat or sham treatment, rats underwent an intraperitoneal glucose tolerance test (IPGTT). Following an overnight fast, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) and injected with a glucose load of 2 g/kg body wt. Tail blood was removed every 30 min and assessed for blood using a glucometer and the manufacturer's test strips (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN, USA). Blood was allowed to clot for 30 min on ice, spun at 3,000 g for 60 min at 4°C, and serum drawn off and frozen at -80°C. Serum was analyzed for concentration of insulin using an insulin ELISA (Alpco, Salem, NH, USA). Upon sacrifice, blood was also collected, allowed to clot for 30 min on ice, spun at 3,000 g for 60 min at 4°C, and serum drawn off and frozen at -80°C. Serum triglycerides and NEFAs were determined by colorimetric assays using the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA and Wako Diagnostics, Richmond, VA, USA, respectively).

Glucose transport. Insulin-stimulated glucose transport into EDL and soleus muscle strips was determined as previously described (152, 154, 155, 538). After dissection, muscle strips were placed in vials in a shaking incubator (35°C) for 60 min containing Krebs-Henseleit bicarbonate (KHB) buffer with 8 mmol/l glucose and 32 mmol/l mannitol. Muscle strips were transferred to new vials containing 2 ml of KHB and 40 mmol/l mannitol, with or without insulin (1 mU/ml) for 30 min at 29°C. Muscle strips were again transferred to new vials containing 2 ml of KHB and 4 mM 2-[1,2-³H]deoxyglucose (1.5 µCi/ml) and 36 mmol/l [¹⁴C]mannitol (0.2

$\mu\text{Ci/ml}$), with or without insulin (1 mU/ml) for 20 min. During all incubation steps, muscle strips were exposed to a gas phase of 95% O_2 -5% CO_2 . Finally, muscle strips were blotted, clamp-frozen at -80°C , and processed for determination of intracellular and extracellular space accumulation of 2-deoxyglucose accumulation.

Intramuscular triglyceride content. Intramuscular triacylglycerol concentration was determined based on the methods by Frayn and Maycock (123). The tibialis anterior, consisting of mostly glycolytic muscle fibers (94), was homogenized in 3 ml of 2:1 chloroform:methanol, transferred to 13x100 mm borosilicate glass tubes, vortexed, and incubated overnight at 4°C . The following day, 3 ml of 4 mmol/l MgCl_2 was added to each tube, vortexed, and centrifuged at 1,000 g for 1 hr at 4°C . The bottom organic layer (1.5 ml) was drawn off and placed into clean borosilicate glass tubes, allowed to dry overnight, reconstituted with 500 μl of ethanolic KOH, and heated at 75°C for 20 min. Following heating, 1 ml of 0.15 mmol/l MgSO_4 was added to each tube, centrifuged at 1,000 g for 1 hr at 4°C , and supernatant removed and assayed for triglyceride and free glycerol concentration using a commercially available colorimetric assay (F6428, Sigma, St. Louis, MO). Liver was processed similarly except that after drying overnight, samples were reconstituted in butanol – Triton X-110 and assayed directly afterwards (402).

Adipose tissue imaging. Epididymal white adipose tissue was fixed overnight in 4% paraformaldehyde, placed in 70% ethanol for 48-72 hr, processed and paraffin embedded. Ten μm sections were placed on slides and subsequently hematoxylin and eosin (H & E) stained. Images were taken on a Nikon 80i microscope and images quantified using Image J.

Western blotting. Muscles, adipose tissue, and liver were processed for Western blotting by methods previously described (154, 155, 412). Briefly, muscle and liver tissue were

homogenized in a 12:1 (volume-to-weight) ratio of ice-cold cell extraction buffer containing 10 mmol/l Tris·HCl (pH 7.4); 100 mmol/l NaCl; 1 mmol/l each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mmol/l Na_3VO_4 ; 20 mmol/l $\text{Na}_4\text{P}_2\text{O}_7$; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250 μl /5 ml protease inhibitor cocktail. Adipose tissue was homogenized in a 2:1 (volume-to-weight) ratio of ice-cold cell extraction buffer and was additionally sonicated for 3-5 s. Homogenates were rotated for 30 min at 4°C, and then centrifuged for 20 min at 3,000 rpm at 4°C. The supernatant was removed and protein concentration determined by Bradford assay. Samples were diluted in HES buffer and Laemmli buffer containing 100 mmol/l dithiothreitol (DDT) (Thermo Scientific, Rockford, IL, USA) based on protein concentration to generate samples containing equal concentration of protein. Samples were heated in a boiling water bath 5 min.

Protein (30-80 μg) was separated on SDS-PAGE gels, followed by a wet transfer to a nitrocellulose membrane for 60-90 min at 200-400 mA. Membranes were blocked in Tris-buffered saline (TBS), 0.1% Tween 20 (TBST), and 5% nonfat dry milk or 5% bovine serum albumin (BSA) followed by incubation with the appropriate primary antibodies. Following three brief washes with TBST, blots were incubated with an appropriate HRP-conjugated secondary antibody in TBST 1% nonfat dry milk at a concentration of 1:5,000 or 1:10,000 for 1-2 hr at room temperature. Blots were then washed twice with TBST and once with TBS, dried, and visualized by Enhanced chemiluminescence (ECL). Bands were quantified using Image J densitometry. Blots were then stripped for 15-20 min at 55°C in buffer containing 62.5 mmol/l Tris·HCl, 2% SDS, and 100 mmol/l 2-mercaptoethanol and re-probed for α -tubulin or β -actin as a loading control.

Primary antibodies used included HSP72 (Cat # SPA-810, Enzo Life Sciences, Farmingdale, NY, USA), phospho-HSP27 (Ser-82) (Cat # 905-642, Stressgen, Victoria, British Columbia, Canada), HSP25 (Cat # SPA-801, Stressgen), HSP60 (Cat # SPA-807, Stressgen), PGC-1 α (Cat # 516557, Cal-Biochem, Darmstadt, Germany), UCP-1 (Cat # 662045), LC3B (Cat # 2775, Cell Signaling Technology, Inc., Danvers, MA, USA), p62 (Cat # 5114, Cell Signaling), α -tubulin (Cat # ab7291, Abcam, Cambridge, MA, USA), and β -actin HRP-conjugated (Cat # ab20272, Abcam). Secondary antibodies used included goat anti-mouse (Cat # 170-5047, BioRad, Hercules, CA, USA) and donkey anti-rabbit (Jackson, Immuno-Research, Inc., West Grove, PA, USA).

Statistical analyses. Results are presented as mean \pm SEM. Statistical significance was set at $P < 0.05$. Analysis was performed using Sigma Plot for Windows, version 12.0 (Systat Software Inc., Chicago, IL, USA). Data were compared by one-way ANOVA or two-way ANOVA with LSD post-hoc differences performed where appropriate. Changes in blood glucose and insulin during glucose tolerance testing were analyzed by two-way repeated measures ANOVA with LSD post-hoc differences performed where appropriate, as well as pairwise comparisons made by one-way ANOVA. Where raw values did not meet the ANOVA assumptions of equal variance or normal distribution, values were logarithmically or square root transformed. Raw data are presented in all figures.

3.4. RESULTS

At the end of the initial 8 wk period of high-fat feeding, body weight (Sham: 498.8 ± 19.1 g, Heat: 492.1 ± 15.7 g, $P = 0.8$) and daily food intake (Sham: 19.2 ± 0.8 g/d, Heat: 19.4 ± 1.1

g/d, $P = 0.92$) were not significantly different between animals placed into treatment groups. During the treatment period consisting of the last 7 wk with continued high-fat feeding, there was a strong trend for heat treated rats to gain less weight during the treatment period compared to sham treated animals ($P = 0.052$) (**Figure 8A**). Food intake was not significantly different during the treatment period ($P = 0.44$) (**Figure 8B**). Energy efficiency, calculated as the change in body weight divided by the kilocalories consumed during this period, was significantly lower in the heat treated rats compared to sham treated ($P = 0.042$) (**Figure 8C**). Coinciding with modest changes in body weight, the weight of the eWAT (Sham: 17.1 ± 2.0 g, Heat: 14.3 ± 1.2 g, $P = 0.27$), subcutaneous white adipose tissue (SCAT) (Sham: 9.3 ± 1.5 g, Heat: 6.7 ± 1.1 g, $P = 0.19$), and brown adipose tissue (BAT) (Sham: 729.4 ± 67.3 mg, Heat: 700.0 ± 38.7 mg, $P = 0.70$) were not significantly different. Although adipose tissue mass was not significantly different, adipocyte cell area in the eWAT was significantly lower in heat treated rats compared to sham treated ($P < 0.01$) (**Figure 8D & 8E**).

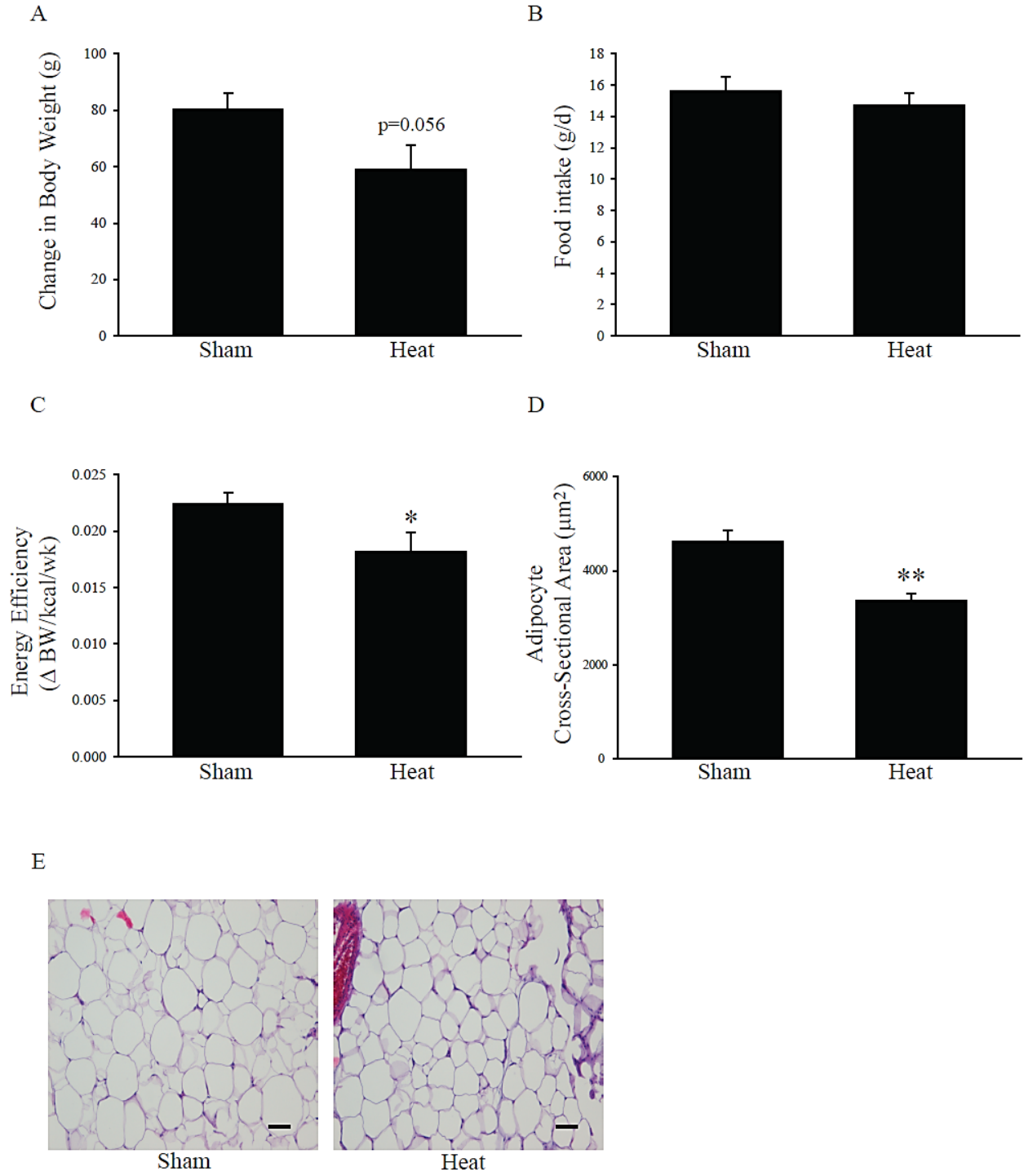
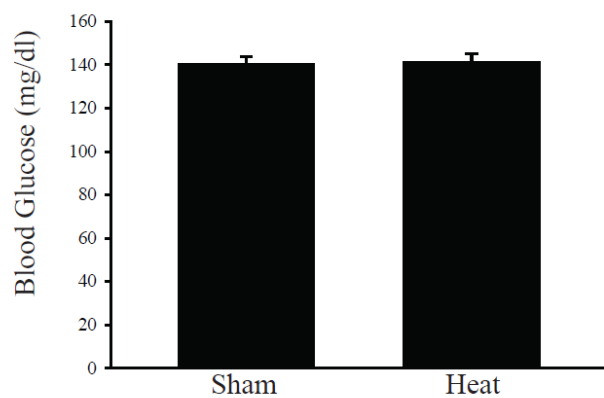


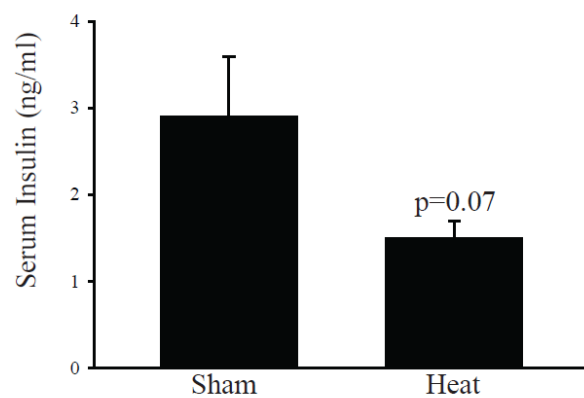
Figure 8. Body weight related changes following weekly heat treatment in rats fed a HFD. (A) Change in body weight, (B) food intake, (C) energy efficiency, and (D) cross-sectional area of adipocytes from eWAT in rats fed a HFD for 15 wk and receiving weekly *in vivo* sham (37°C, 20 min) or heat treatment (41°C, 20 min) during the last 7 wk of the HFD. (E) Representative images of 10 µm thick sections of eWAT and H&E stained. Bar represents 50 µm. * $P < 0.05$, ** $P < 0.01$ denotes a significant difference between groups determined by one-way ANOVA with LSD post hoc analysis performed when appropriate. Values are mean \pm SEM. N = 8-9 animals/group.

Whole-body insulin resistance has been consistently shown after just 3-6 wk of high-fat feeding (262, 358, 462, 468). Fasting blood glucose concentration was not significantly different between sham and heat treated rats (**Figure 9A**), and fasting insulin concentration was only modestly reduced in heat treated animals compared to sham treated animals ($P = 0.07$) (**Figure 9B**). Following an intraperitoneal injection of glucose one wk prior to the final heat or sham treatment, heat treated rats had significantly lower blood glucose concentrations compared to sham treated (main effect of treatment $P < 0.05$) (**Figure 9C**), as well as lower glucose area under the curve (AUC) values ($P = 0.014$) (**Figure 9D**). Insulin concentration was significantly lower 30 min following glucose injection in heat treat rats compared to sham treated, but was not significantly different 60 min, 90 min, or 120 min following injection (**Figure 9E**). Insulin AUC values were not significantly different between heat and sham treated rats (**Figure 9F**). Serum triglycerides were not significantly different between sham (120.7 ± 11.3 mg/dl) and heat treated rats (114.3 ± 17.1 mg/dl, $P = 0.77$), and serum NEFA concentration were not significantly different between sham (0.469 ± 0.04 mmol/l) and heat treated animals (0.421 ± 0.04 mmol/l, $P = 0.4$).

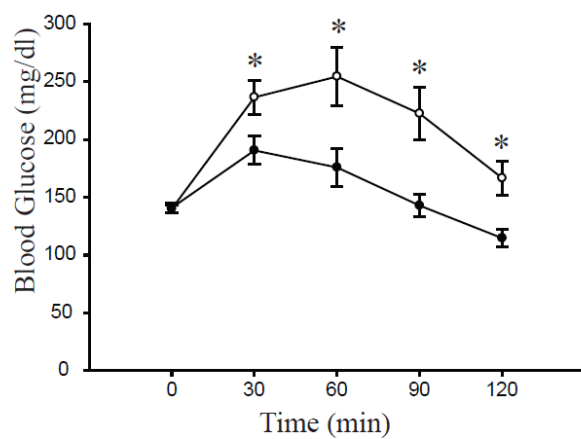
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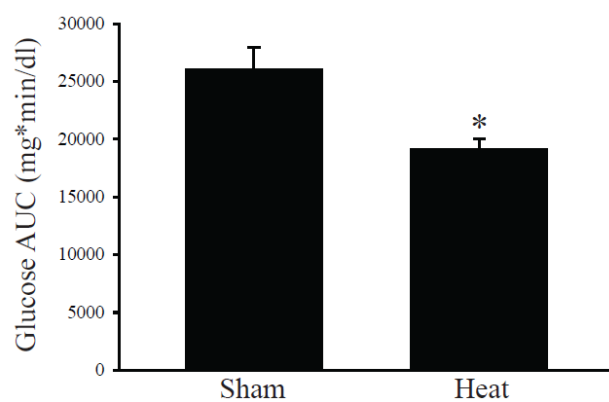
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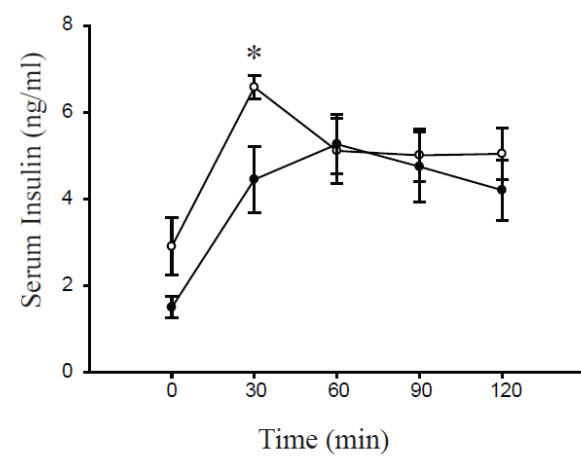
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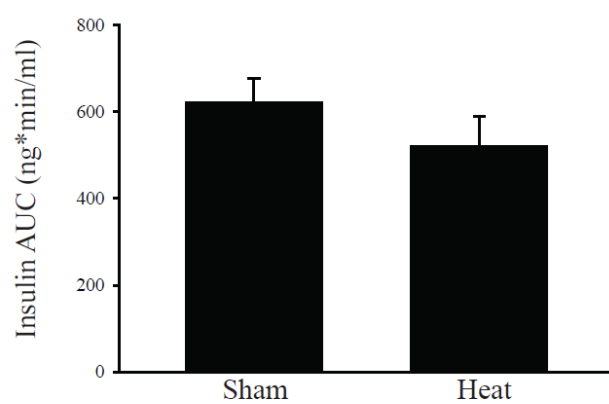


Figure 9. Heat treatment improves glucose tolerance in rats fed a HFD. (A) Fasting blood glucose and (B) serum insulin concentration in rats fed a HFD for 15 wk and receiving weekly *in vivo* sham (37°C, 20 min) or heat treatment (41°C, 20 min) during the last 7 wk of the HFD. Blood glucose (C) and insulin concentrations (E) in response to an intraperitoneal (i.p.) glucose injection, and AUC of glucose (D) and AUC (F) of insulin following i.p. glucose injection. Rats were fasted overnight one week prior to sacrifice and were injected with a glucose load of 2 g/kg body weight intraperitoneally. Blood glucose was measured prior to, and 30, 60, 90, and 120 min following injection using a glucometer. Heat treatment reduced blood glucose response to i.p glucose injection determined by two-way ANOVA (main effect of treatment). * $P < 0.05$ denotes significantly different between treatment groups determined by one-way ANOVA with LSD post hoc analysis performed where appropriate. Values are mean \pm SEM. N = 8-9 animals/group.

Skeletal muscle responses to heat treatment. The EDL is composed of primarily glycolytic type II muscle fibers while the soleus is composed primarily of oxidative type I muscle fibers (94). In the EDL of heat treated rats, we observed significantly greater insulin-stimulated glucose uptake compared to sham treated rats ($P < 0.05$) (**Figure 10A**). In the soleus, we did not observe significant differences in insulin-stimulated glucose uptake between heat and sham treated rats (data not shown). We observed that in the tibialis anterior, another primarily glycolytic muscle (94), triglyceride content was significantly lower in heat treated rats compared to sham treated ($P < 0.05$) (**Figure 10B**).

HSP72 and HSP60 levels were significantly greater in the EDL of heat treated rats compared to sham treated (**Figure 10C & 10D**), but HSP72 and HSP60 levels were not significantly different between heat and sham treated rats in the soleus (**Figure 10F & 10G**). Phosphorylation of HSP25 (Ser-82) and total HSP25 levels were not significantly different between heat and sham treated rats in the EDL or soleus (data not shown). PGC-1 α levels were significantly lower in the soleus of heat treated animals ($P = 0.02$) (**Figure 10H**), and there was a trend for PGC-1 α levels to decrease in the EDL ($P = 0.08$) (**Figure 10E**).

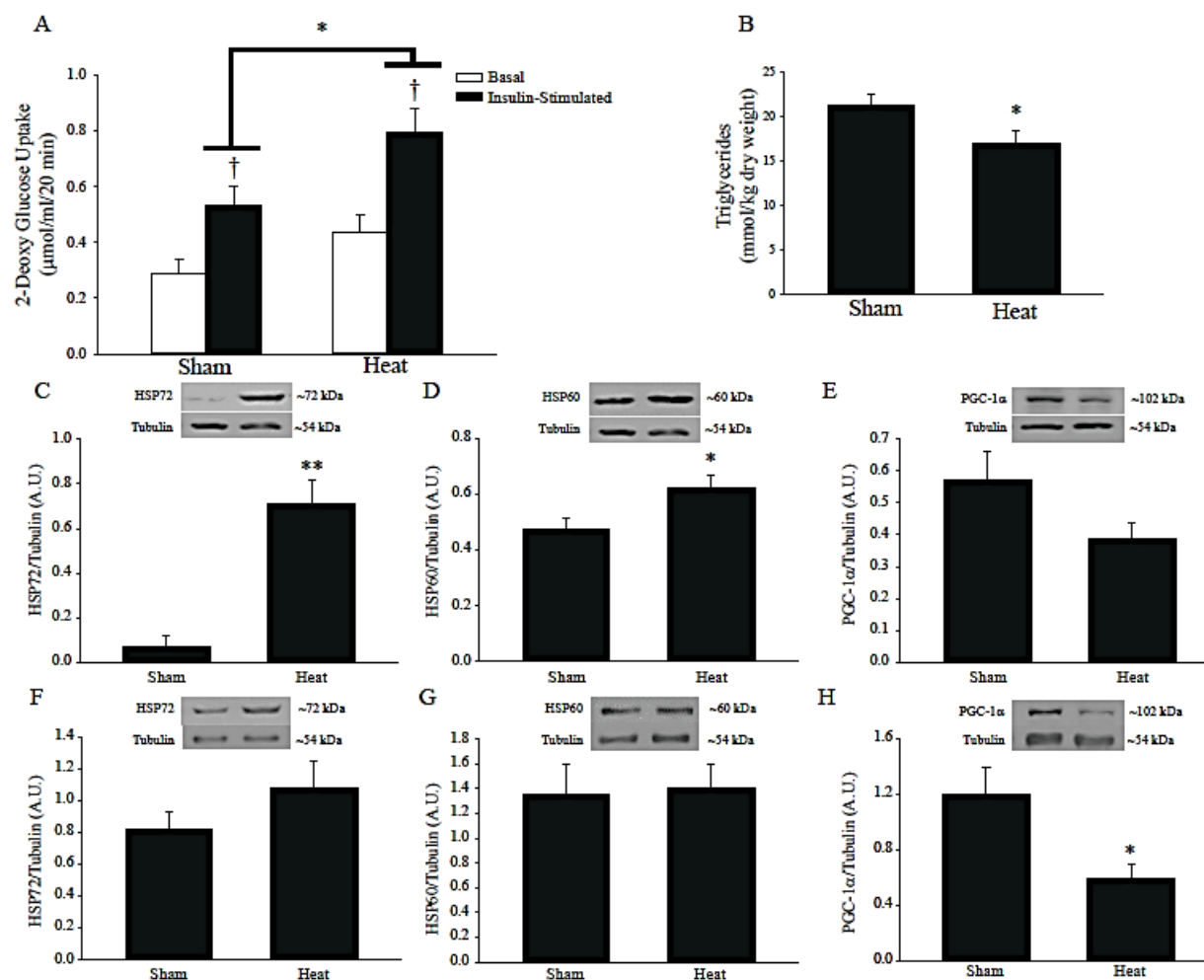


Figure 10. Heat treatment improves insulin-stimulated glucose uptake in the EDL of rats fed a HFD. (A) Insulin-stimulated glucose uptake in the EDL muscle in sham and heat treated rats fed a HFD for 15 wk and receiving weekly *in vivo* sham (37°C, 20 min) or heat treatment (41°C, 20 min) during the last 7 wk of the HFD. Insulin-stimulated glucose uptake was determined by incubating muscles in the presence or absence of 1 mU/ml insulin for exactly 20 min. (B) Triglyceride content in tibialis anterior in sham and heat treated rats fed a HFD. HSP72, HSP60, and PGC-1α expression in the EDL (C-E) and soleus (F-H), respectively. * $P < 0.05$, ** $P < 0.05$ denotes significantly different between treatment groups; † $P < 0.05$ denotes insulin-stimulated glucose uptake is significantly greater than basal determined by one-way ANOVA with LSD post hoc analysis performed where appropriate. Values are mean \pm SEM. N = 6-9 animals/group.

Hepatic responses to heat treatment. Similar to skeletal muscle, hepatic triglyceride content was significantly lower in heat treated, high-fat fed rats compared to sham treated rats (**Figure 11A**). Hepatic HSP72 and HSP25 expression were significantly greater in heat treated animals compared to sham treated animals (**Figure 11B & 11C**). As markers of autophagy, LC3 is lipidated to form LC3-II when autophagosomes are forming while p62 is degraded, and LC3-II and p62 are considered markers of increased autophagy (397). LC3-II levels were significantly greater in heat treated rats fed a HFD compared to sham treated (**Figure 11D**), and p62 levels were significantly lower in heat treated rats compared to sham treated (**Figure 11E**).

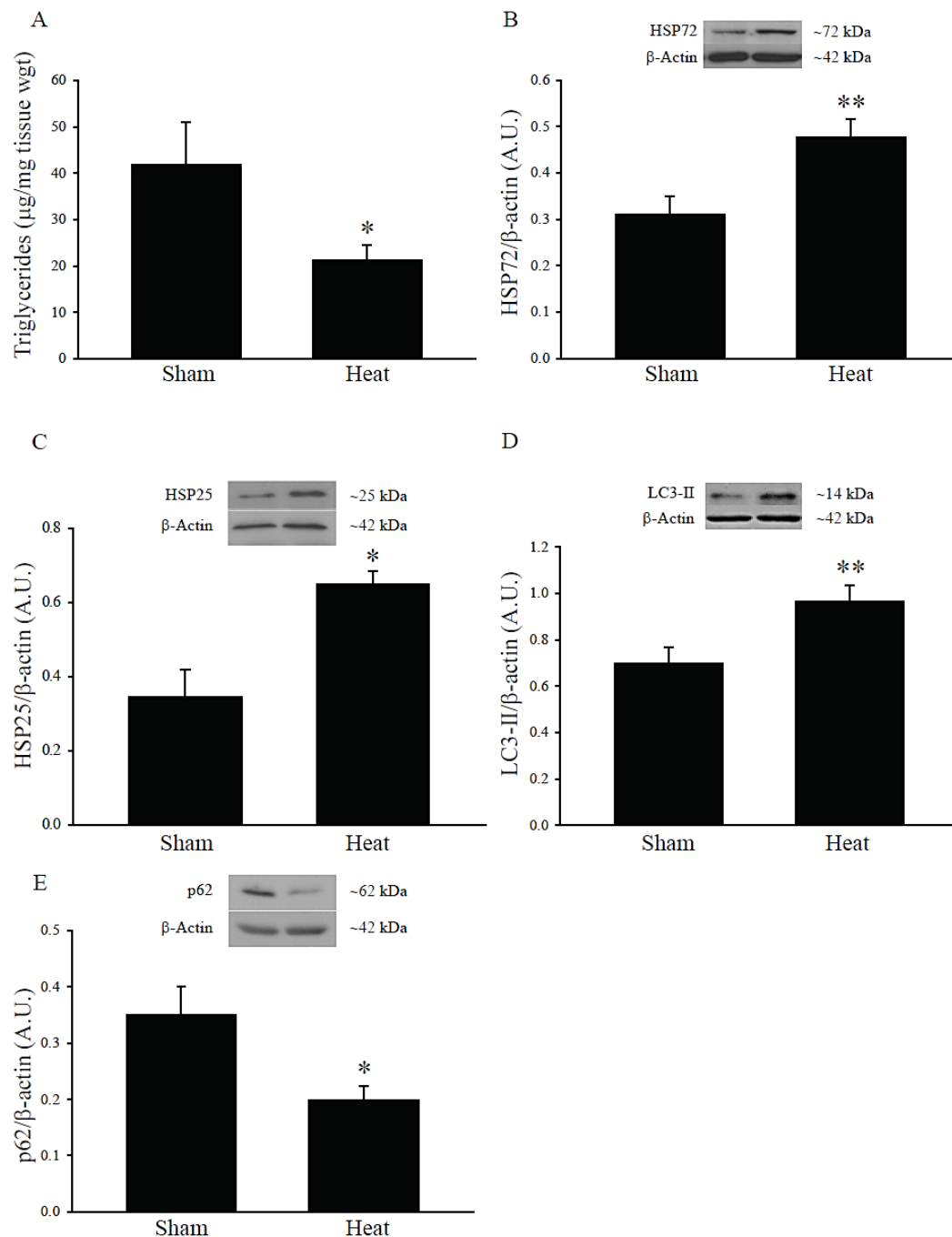


Figure 11. Heat treatment reduces triglyceride content and increased autophagy in rats fed a HFD. (A) Triglyceride content, (B) HSP72 expression, (C) HSP25 expression, and content of LC3-II (D) and p62 (E) in sham and heat treated rats fed a HFD for 15 wk and receiving weekly *in vivo* sham (37°C, 20 min) or heat treatment (41°C, 20 min) during the last 7 wk of the HFD. * $P < 0.05$, ** $P < 0.05$ denotes significantly different between treatment groups determined by one-way ANOVA with LSD post hoc analysis performed where appropriate. Values are mean \pm SEM. N = 8-9 animals/group.

Adipose tissue HSP72 and PGC-1 α are increased following heat treatment. HSP72 expression was significantly greater in the eWAT and SCAT in heat treated animals compared to sham treated animals (main effect of treatment $P = 0.011$) (**Figure 12A**). There were not differences between eWAT and SCAT in HSP72 expression in these HFD fed rats (main effect of depot $P > 0.05$) (**Figure 12A**). PGC-1 α expression was significantly greater in heat treated rats compared to sham treated rats (main effect of treatment $P = 0.007$) (**Figure 12B**). Furthermore, PGC-1 α levels were significantly greater in the SCAT compared to the eWAT (main effect of depot $P = 0.018$) (**Figure 12B**). Uncoupling protein-1 (UCP-1), which uncouples mitochondrial respiration and has been shown to be induced in WAT by exercise training and adrenergic receptor activation (92, 224, 415), was significantly increased in eWAT and SCAT (main effect of treatment $P < 0.05$) (**Figure 12C**).

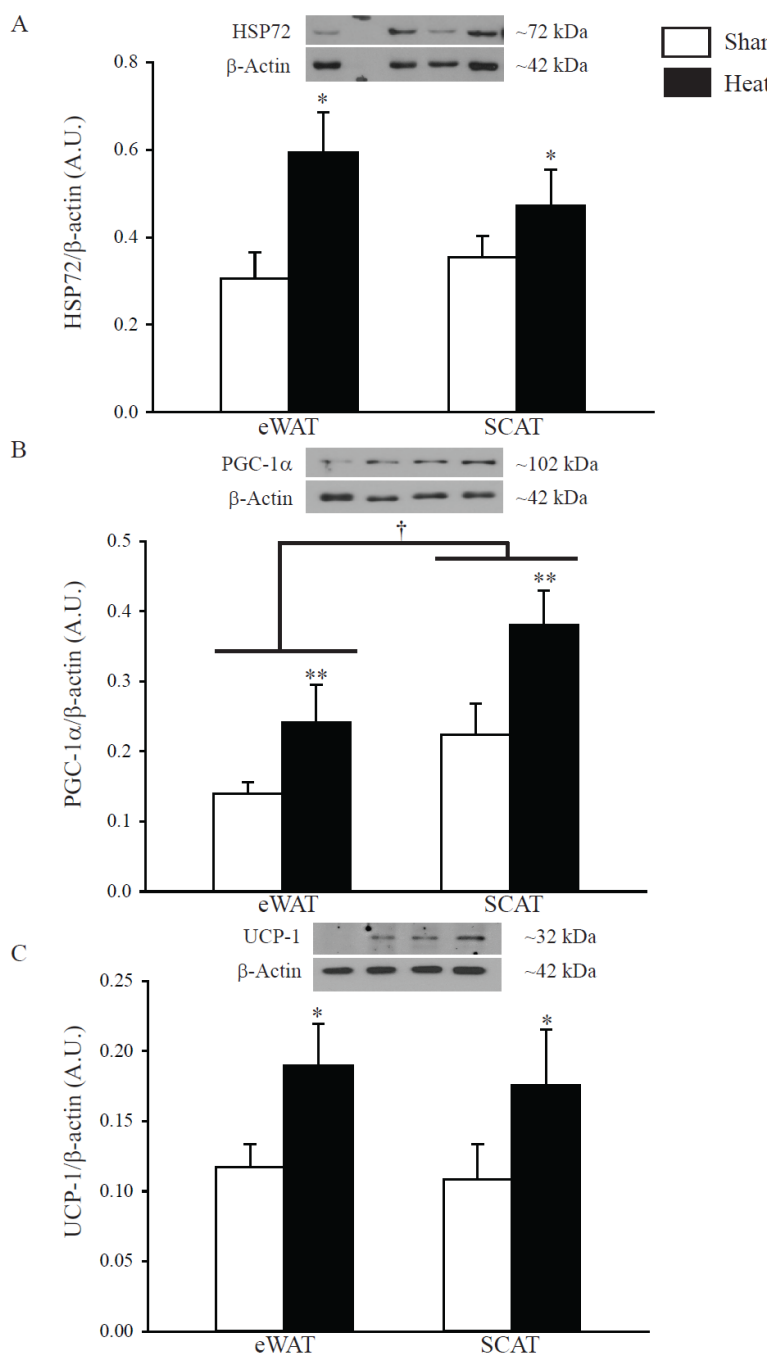


Figure 12. Heat treatment increases HSP72, PGC-1 α , and UCP-1 levels in WAT of rats fed a HFD. (A) HSP72, (B) PGC-1 α , and (C) UCP-1 expression in eWAT and SCAT of sham and heat treated rats fed a HFD for 15 wk and receiving weekly *in vivo* sham (37°C, 20 min) or heat treatment (41°C, 20 min) during the last 7 wk of the HFD. * $P < 0.05$, ** $P < 0.05$ denotes significantly significant main effect of heat treatment, † $P < 0.05$ denotes significant main effect of depot determined by a two-way ANOVA with LSD post hoc analysis performed where appropriate. Values are mean \pm SEM. N = 8-9 animals/group.

3.5. DISCUSSION

Previously we showed that 12 wk of weekly *in vivo* heat treatment prevented HFD induced whole-body and skeletal muscle insulin resistance (154). In the present study, we expand upon these findings by showing that after 8 wk of high-fat feeding, weekly *in vivo* heat treatment of high-fat fed rats reduces glucose intolerance and increases insulin sensitivity in primarily glycolytic skeletal muscle. Furthermore, we show that weekly *in vivo* heat treatment reduces lipid storage and improves the metabolic profile of other metabolic tissues that regulate glucose homeostasis including the liver and white adipose tissue. These are the first findings to indicate the ability of heat treatment to reverse the damaging effects of a prior HFD.

Whole-body and skeletal muscle adaptation to heat treatments in high-fat fed rats. *In vivo* heat treatments, pharmacological induction of HSP72, and skeletal muscle HSP72 transgenic overexpression have been shown to improve glucose homeostasis, reduce insulin resistance, and reduce adipose tissue mass in a number of diabetic rodent models (28, 67, 154, 256). Here we also show that heat treatment improved glucose intolerance and increased insulin-stimulated glucose uptake in glycolytic skeletal muscle of rats that were fed a HFD 8 wk prior to initiation of heat treatments. Whole-body and skeletal muscle insulin resistance has been consistently shown after just 3-6 wk of high-fat feeding (262, 358, 462, 468). The inability of heat treatment to increase insulin-stimulated glucose uptake in the soleus may relate to the established insulin resistance prior to initiation of weekly treatment has blunted the induction of HSPs. A blunted induction of HSPs has been shown following heat or exercise in diabetic animals (17, 236).

High-fat feeding in rats increases skeletal muscle mitochondrial protein levels to compensate for excess dietary lipids (161, 495). Heat treatment *in vivo* and *in vitro*, as well as transgenic overexpression of HSP72 in skeletal muscle, increases mitochondrial enzyme activity

and increases mitochondrial content (59, 67, 154, 173, 293, 471). The ablation of HSP72 in mice results in whole-body and skeletal muscle insulin resistance as well as accumulation of dysmorphic mitochondria (101). Recently HSP72 has been shown to regulate mitophagy – the autophagic process of removing damaged mitochondria (101). In the present study and our previous study (154), we observed lower PGC-1 α expression in skeletal muscle of high-fat fed heat treated rats. This may support the hypothesis that mitochondrial quality is improved by the induction of HSP72 as there is a lower requirement for mitochondrial biogenesis reflected by lower PGC-1 α expression following heat treatment.

Increased lipid storage in the skeletal muscle has been shown to generate secondary messengers (i.e., DAGs and ceramides) that inhibit insulin signaling (340, 342). In the present study, we observed lower triglyceride content in glycolytic skeletal muscle of heat treated rats fed a HFD in association with increased insulin-stimulated glucose uptake. The observed reduction may imply that excess dietary lipids are being oxidized to a greater extent in the skeletal muscle of heat treated rats. Although triglycerides themselves may not inhibit insulin signaling, intermediates of fatty acid metabolism such as DAGs, ceramides, and long-chain acyl carnitines can activate stress kinases including JNK, IKK β , and PKC inhibiting insulin signaling (5, 6, 147, 340, 342). We and others have shown that induction of HSP72 by heat treatment, pharmacologically, or genetic modulation inhibits JNK activation and may be a mechanism by which HSP72 improves insulin responsiveness (3, 67, 154, 155, 368), although recently this has been found to be situationally dependent (173). As skeletal muscle is responsible for 75%-80% of post-prandial glucose disposal (115), we had concentrated on the skeletal muscle following heat treatment as responsible for the observed metabolic adaptations in our previous study. In the present study, we observed that in heat treated rats have smaller adipocytes in the eWAT and

reduced triglyceride storage in the liver, which may also be a means by which whole-body glucose intolerance is improved.

Adipose tissue responses to heat treatment in rats fed a HFD. In the present study, we showed that in rats fed a HFD, weight gain during the treatment period was slowed by weekly heat treatment and energy efficiency was reduced indicating that energy utilization had been altered. Adipocyte size was reduced by weekly heat treatment in the eWAT although fat pad mass was not significantly reduced by weekly heat treatments. Our results agree with prior studies that heat treatment, transgenic overexpression, or GGA administration to induce HSP72 levels reduces WAT mass and adipocyte size (3, 67, 154). Adipocytes are known to play a large role in whole-body glucose homeostasis (415), and smaller adipocytes are generally more insulin sensitive (15). We have previously shown that a single *in vivo* heat treatment increases insulin responsiveness in the eWAT (412), and improved insulin responsiveness may represent the early adaptation of the adipose tissue to heat treatments. Although it cannot be ruled out that heat treatment changed lipogenic and lipolytic protein expression, other mechanism may play a role. Heat treatment has been shown to modulate membrane fluidity (483, 484, 490), thus the fluidity of the lipid droplet membrane in the adipocytes themselves may be modulated in a fashion that results in reduced triglyceride storage.

HSP72 levels increased in both the eWAT and SCAT of heat treated rats fed a HFD, without differences between depots. Previously, we had noted in male Wistar rats 9-10 wk younger than used in the present study that constitutive HSP72 levels are greater in the metabolic WAT depots (i.e., epididymal and retroperitoneal) compared to SCAT (412), which has been observed elsewhere (383, 421, 458). The rats used here were older and fed a HFD for 15 wk, and

we did not observe depot differences in HSP72 expression. A few reports have noted that HSP levels are greater in omental and SCAT depots of obese humans compared to lean counterparts (97, 382, 383). Thus constitutive HSP levels in these rats may have increased in conjunction with high-fat feeding and adiposity. We also noted previously that the induction of HSP72 was greatest in the SCAT following heat treatment, but here the induction of HSP72 in the SCAT appeared to be blunted as it was not different than in the eWAT. The heat shock response has been shown to be either blunted or enhanced during obesity and diabetes (17, 236, 346), and the blunted SCAT induction of HSP72 may be a result of the high-fat feeding induced insulin resistance. The activation of JNK and other stress kinases such as ERK and GSK3 can inhibit HSF-1 activation, the primary transcription factor responsible for inducing the transcription of HSPs following heat treatment (9, 329). As JNK activation in the adipose tissue occurs during obesity (519), HSF-1 inhibition may be responsible for the blunted heat shock response observed in the SCAT here.

Hepatic response to heat treatment in rats fed a high-fat diet. Little is known regarding the role of HSPs in preventing hepatic steatosis and NAFLD. As excess lipid accumulation in the liver is a hallmark of NAFLD, reductions in triglyceride storage observed here represents a potential treatment modality that has not been widely explored. Adachi *et al.* reported that induction of HSP72 by administration of GGA ameliorated hepatic insulin resistance and reduced JNK phosphorylation (3), but hepatic triglyceride content was not measured. Reports using mild electrical stimulus coupled with heat stress have shown reduced triglyceride storage, reduced inflammation, and increased insulin responsiveness in the liver of high-fat fed mice and *db/db* mice (330, 331). In these reports, HSP levels were not measured. Here we observed that both HSP72 and HSP25 levels are induced by weekly heat treatment in rats fed a HFD in

conjunction with lower triglyceride content. Thus more research is needed to determine if HSP72 or HSP25 have a direct role in the protection against steatosis.

Autophagy is the sequential process of targeting and degrading organelles. After the initiation of autophagy, during the formation of the autophagosome and its fusion with the lysosome, LC3 is lipidated forming LC3-II resulting in degradation of p62. Increased LC3-II and decreased p62 levels potentially indicate that the autophagy degradation pathway has become more active (99, 429). In the present study, heat treatment increased the content of LC3-II and reduced p62 levels in high-fat fed rats. Similarly, transgenic overexpression of HSP72 in skeletal muscle enhances autophagy during fasting (173). Autophagy is essential for mobilizing lipids stored in the liver – termed lipophagy (451), and may indicate that lipids are being mobilized from the lipid droplets for oxidation in the liver. Additionally, LC3-II also accumulates during increased mitophagy (99), and as HSP72 has been shown to regulate mitophagy to clear damaged mitochondria, improved mitochondrial quality may be another mechanism by which triglyceride levels are reduced.

CONCLUSION

Heat treatments improve glucose intolerance and skeletal muscle insulin resistance in a rodent model of established obesity and insulin resistance induced by high fat feeding. Weekly heat treatments also improve the metabolic profile of the liver and adipose tissue. Thus, treatments that induce the expression of HSP72 should be explored as treatment options for other metabolic conditions, including NAFLD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

CHAPTER 4

Decreased heat shock protein expression underlies susceptibility to short term metabolic insult in rats selectively bred as low capacity runners

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Manuscript in preparation

4.1. ABSTRACT

Heat shock protein (HSP) expression positively correlates with oxidative capacity and conversely, protein expression of HSP72 is reduced in patients with type 2 diabetes. In the current study, we examined whether constitutive HSP expression is associated with aerobic capacity and susceptibility to metabolic disease in rats bred to be low capacity runners (LCR) and high capacity runners (HCR). We also tested the ability of HSP induction to restore insulin sensitivity following a 3 day high-fat diet (HFD) challenge in LCR and HCR rats. HCR and LCR rats were fed a HFD (45% of kilocalories from fat) or chow diet (10% of kilocalories from fat) for 3 days and then received either a single *in vivo* heat treatment (HT, 41°C, 20 min) or sham treatment (ST). 24 hr following HT or ST, glycolytic (extensor digitorum longus, EDL) and oxidative (soleus) muscles were removed to measure insulin-stimulated glucose uptake and blood, liver and adipose tissues were harvested and stored at -80°C. HT restored insulin sensitivity primarily in the EDL muscle of LCR rats following a 3 d HFD challenge, while muscle insulin sensitivity was unchanged in HCR rats with diet or HT. Induction of HSP72 following HT occurred in the EDL and liver of LCR and HCR rats, but not in the soleus of LCR rats. The induction of HSP72 in the EDL and liver, and HSP25 in the liver, was blunted in LCR rats following a 3 d HFD, while HSP72 levels were maintained in HCR rats. Constitutive HSP72 and heat shock factor-1 (HSF-1) expression was lower in EDL muscle of LCR rats compared to HCR rats, and HSP25 expression was lower in the liver of LCR rats compared with HCR rats. The mitochondrial HSP, HSP60, and other key mitochondrial proteins were lower in the EDL muscle compared to the soleus muscle of LCR rats. Finally, protein content of LC3-II, a marker of autophagy, increased in the skeletal muscle of HCR rats following a 3 d HFD challenge, but did not change in LCR rats. In summary, a single heat treatment lowered blood glucose levels,

restored skeletal muscle insulin sensitivity and decreased adipocyte size and liver triglyceride content following an acute dietary challenge in rats with low aerobic capacity. Lower constitutive levels of HSP and mitochondrial proteins were associated with increased susceptibility to metabolic insult primarily in the glycolytic muscles from LCR rats.

Key words: aerobic capacity, insulin resistance, type 2 diabetes, heat shock proteins

4.2. INTRODUCTION

The prevalence of obesity, metabolic syndrome, and type 2 diabetes continues to rise toward epidemic proportions. Low aerobic capacity is a strong independent predictor of metabolic syndrome, type 2 diabetes, cardiovascular disease, and all-cause mortality - even when traditional risk factors are considered such as smoking and obesity (69, 253, 255, 273, 344). The mechanisms underlying the relationship between low intrinsic aerobic capacity and susceptibility to chronic metabolic disease remain largely unknown. Interestingly, an estimated 50%-70% of an individual's aerobic capacity, and thus disease risk, can be attributed to inheritable traits (46). HSPs strongly correlate with oxidative capacity in skeletal muscle and adipose tissue (45, 296, 412), and may also underlie innate differences in susceptibility to metabolic disease.

The heat shock response is a highly conserved defense system to combat cellular and oxidative stress (9, 329), and involves induction of a family of HSPs identified by molecular weight (329). HSP72 is of great interest in relation to metabolic disease as it is highly induced in response to stress and with endurance exercise (329, 334). Kurucz *et al.* first demonstrated that HSP72 expression was markedly decreased in skeletal muscle of insulin resistant and type 2 diabetic patients (269). Subsequent studies showed that heat treatment, transgenic overexpression of HSP72, and pharmacological induction of HSP72 effectively prevent high fat diet-induced glucose intolerance and skeletal muscle insulin resistance (3, 67, 154, 173, 291). Overexpression of skeletal muscle HSP72 in mice has been shown to increase endurance running capacity nearly two-fold and increase mitochondrial content by 50% (173). We hypothesize that the ability of HSPs to improve glucose homeostasis and increase oxidative capacity in skeletal muscle may provide protection against short-term metabolic insult.

The purpose of this study was two-fold. First, to determine whether constitutive HSP expression is associated with aerobic capacity and susceptibility to metabolic disease in rats bred to be low capacity runners (LCR) and high capacity runners (HCR). LCR rats display symptoms of metabolic disease including glucose intolerance, skeletal muscle insulin resistance, and increased hepatic triglyceride storage (286, 332, 355, 357, 476, 525). To date, the role of HSPs in metabolic disease susceptibility of LCRs or metabolic protection of HCRs has not been examined. Metabolic disturbances in LCR rats can be further exacerbated by high-fat feeding, while HCR rats are generally protected from high-fat feeding induced insulin resistance (285, 286, 332, 355, 407, 476, 525). As a result, the second purpose of this study was to test the ability of HSP induction via heat treatment to restore insulin sensitivity following an acute high fat diet challenge in LCR rats. Our results demonstrate beneficial metabolic effects of heat treatment in multiple tissues.

4.3. METHODS

Animal strains. The development of LCR and HCR rats has been previously described elsewhere (251, 355). Briefly, a founder population of genetically heterogeneous rats from the N:NIH stock (National Institutes of Health) were bred by two-way artificial selection to produce rat strains differing in inherent endurance running capacity. The 13 lowest and 13 highest running capacity rats from each sex were selected from the founder population and randomly paired for mating. The animal protocols were approved by the Institutional Animal Care and Use Committees at the University of Kansas Medical Center, University of Michigan, and University of Missouri, as well as, the Subcommittee for Animal Safety at the Harry S. Truman Memorial VA Hospital.

Approximately six- to seven-month old male LCR and HCR rats from *generations 25 and 30* were used for the current investigation. Animals were housed in a temperature controlled facility with 12:12 h light:dark cycles. The rats were provided standard rat chow and water *ad libitum* upon arrival until the experimental diet was started. All rats were acclimated to the low-fat control diet (D12450B, 10% kcal from fat; Research Diets, New Brunswick, NJ) for ≥ 7 d prior to initiation of the experimental diet period. Rats were then continued on either the low-fat control diet or placed on a high-fat diet (HFD, D12451, 45% of kilocalories from fat; Research Diets) for 3 d. Body weight, feeding, energy consumption, and liver parameters for animals in *generation 30* have been reported elsewhere (332).

LCR and HCR rats from *generation 25* were fed a standard chow diet or a HFD for 3 d. After consuming the diets for 3 d, rats were anesthetized with ketamine-xylazine (80 mg/kg body wt-10 mg/kg body wt) and received a single *in vivo* heat or sham treatment. This generated four treatment groups with HCR/LCR strains: Chow-Sham, Chow-Heat, HFD-Sham, and HFD-Heat. Heat treatment consisted of lower body immersion in a 42°C water bath to gradually raise body temperature to between 41°C and 41.5°C where it was maintained for 20 min (154, 412). Temperature was monitored by a rectal thermometer. Sham treatment consisted of immersion in a 37°C water bath and maintaining body temperature. Twenty-four hours following heat or sham treatment, and following 10-h overnight fast, animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt) and tissues dissected for experimental procedures.

Blood Measures. Blood was obtained by clipping the end of the tail prior to dissection and fasting blood glucose concentration measured using a glucose analyzer and the manufacturer's test strips (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN).

Adipose tissue imaging. Epididymal white adipose tissue (eWAT) was fixed overnight in 4% paraformaldehyde, placed in 70% ethanol for 48-72 hr, processed and paraffin embedded. Ten μm sections were placed on glass slides and subsequently H & E stained. Images were taken on a Nikon 80i microscope and quantified using Image J.

Glucose transport. Insulin-stimulated glucose transport into soleus and EDL muscle strips was determined as previously described (154). After dissection, muscle strips were allowed to recover for 60 min and were subsequently transferred to new vials and incubated with or without insulin (1 mU/ml) for 30 min at 29°C and again transferred to new vials containing 4 mM 2-[1,2- ^3H]deoxyglucose (1.5 $\mu\text{Ci/ml}$) and 36 mM [^{14}C]mannitol (0.2 $\mu\text{Ci/ml}$), with or without insulin (1 mU/ml) for 20 min. Following incubations, muscle strips were blotted, clamp-frozen, and processed for determination of intracellular and extracellular space accumulation of 2-deoxyglucose accumulation and Western blotting.

Intramuscular triglyceride content. Muscle and liver tissue triglyceride content was determined by methods described elsewhere (123, 402). Muscle and liver was processed in 2:1 chloroform:methanol to extract lipids and after being further processed and reconstituted assayed for triglyceride and free glycerol concentration (F6428, Sigma, St. Louis, MO).

Western blotting. Muscles and liver were processed for Western blotting by methods previously described (155, 412). Briefly, tissue was homogenized in ice-cold cell extraction buffer. Homogenates were rotated for 30 min at 4°C, and then centrifuged for 20 min at 3,000 rpm at 4°C. The supernatant was removed and protein concentration determined by Bradford assay. Samples were diluted in HES buffer and Laemmli buffer containing 100 mmol/l dithiothreitol (DDT) (Thermo Scientific, Rockford, IL, USA) based on protein concentration to generate samples containing equal concentration of protein. Protein (40-80 μg) was separated on

SDS-PAGE gels, followed by a wet transfer to a nitrocellulose membrane for 60-90 min at 200-400 mA.

Primary antibodies used included HSP72 (Cat # SPA-810, Enzo Life Sciences, Farmingdale, NY, USA), phospho-HSP27 (Ser-82) (Cat # 905-642, Stressgen, Victoria, British Columbia, Canada), HSP25 (Cat # SPA-801, Stressgen), HSP60 (Cat # SPA-807, Stressgen), HSF-1 (Cat # NB300-730, Novus Biochemicals, Littleton, CO, USA), PGC-1 α (Cat # 516557, CalBiochem, Darmstadt, Germany), MitoProfile Total OXPHOS (Cat # ab110413, Abcam, Cambridge, MA, USA), Sirt1 (Cat # ab75435, Abcam), α -tubulin (Cat # ab7291, Abcam), β -actin HRP-conjugated (Cat # ab20272, Abcam), mtFAM (Cat # sc-23588, Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA), Parkin (Cat # sc-32282, Santa Cruz), phospho-AS160 (Thr642) (Cat # 4288, Cell Signaling Technology, Inc., Danvers, MA, USA), phospho-Akt (Ser473) (Cat # 9271, Cell Signaling), total Akt (Cat # 9272, Cell Signaling), phospho-JNK (Thr183/Tyr185) (Cat # 9251, Cell Signaling), total JNK (Cat # 9252, Cell Signaling), LC3B (Cat # 2775, Cell Signaling), acetylated lysine (Cat # 9441, Cell Signaling), and total AS160 (Cat # ABS54, Millipore Corp., Billerica, MA, USA). Secondary antibodies used included goat anti-rabbit (Cat # sc-2004, Santa Cruz), goat anti-mouse (Cat # 170-5047, BioRad, Hercules, CA, USA), rabbit anti-rat (Cat # A-5795, Sigma, St. Louis, MO, USA), rabbit anti-goat (Cat # A-5420, Sigma), and donkey anti-rabbit (Jackson, Immuno-Research, Inc., West Grove, PA, USA).

Statistical analyses. Results are presented as mean \pm SEM. Statistical significance was set at $P < 0.05$. Analysis was performed using Sigma Plot for Windows, version 12.0 (Systat Software Inc., Chicago, IL, USA). Data were analyzed by one-way or two-way ANOVAs with Fisher's LSD post-hoc comparisons performed where appropriate as noted.

4.4. RESULTS

Heat treatment improves glucose and insulin action. Initial body weight was significantly greater in LCR rats compared to HCR rats, and although both strains increased body weight during the HFD, the LCR rats gained significantly more during the 3 d HFD (**Table 3**). Food and energy intake were significantly greater in LCR rats compared to HCR rats. However, when normalized to body weight, energy intake was not significantly different between LCR/HCR rats following a 3 d HFD. When compared by one-way ANOVA, the food intake and energy intake of HFD-fed LCR rats was significantly greater than HFD-fed HCR rats.

Table 3. Body weight, organ weights, and feeding characteristics for HCR and LCR rats from generation 25 following 3 d of high-fat feeding.

	HCR		LCR	
	Control diet	High-fat diet	Control diet	High-fat diet
Body weight (grams)	364.3 ± 11.2	358.3 ± 10.0	464.9 ± 14.5*	455.6 ± 12.7*
3-d daily weight gain (g/d)	0.5 ± 0.4	1.5 ± 0.4†	1.4 ± 0.2*	5.8 ± 0.5*†#
Food intake (g/d)	15.9 ± 0.6	17.8 ± 0.8†	19.3 ± 0.8*	22.5 ± 0.8*†
Energy intake (kcal/d)	61.4 ± 2.4	84.0 ± 3.5†	74.2 ± 3.2*	106.3 ± 3.7*†
Energy intake (kcal/d)/bw (g)	0.17 ± 0.01	0.24 ± 0.01†	0.16 ± 0.01	0.23 ± 0.01†
Soleus (mg)/bw (g)	0.65 ± 0.02	0.72 ± 0.02†	0.52 ± 0.03*	0.56 ± 0.02*†
EDL (mg)/bw (g)	0.52 ± 0.01	0.51 ± 0.01	0.43 ± 0.01*	0.43 ± 0.01*
Gastrocnemius (mg)/bw (g)	5.94 ± 0.14	5.98 ± 0.09	5.08 ± 0.14*	5.09 ± 0.14*
Tibialis anterior (mg)/bw (g)	1.97 ± 0.03	2.02 ± 0.04	1.65 ± 0.05*	1.66 ± 0.03*
Heart (mg)/bw (g)	2.85 ± 0.07	2.90 ± 0.07	2.63 ± 0.06*	2.60 ± 0.08*

Body weight prior to 3-d high-fat feeding, body weight gain, percentage muscle weights relative to body mass, and feeding characteristics of HCR/LCR rats from *generation 25*. * $P < 0.001$ denotes a significant main effect of strain, † $P < 0.05 - 0.001$ denotes a significant main effect of diet, # $P < 0.001$ denotes a significant strain x diet interaction assessed by two-way ANOVA with Fisher LSD post-hoc comparisons where appropriate.

Fasting blood glucose concentration was significantly greater in LCR rats compared to HCR rats, and heat treatment significantly reduced blood glucose in LCR rats (**Figure 13A**). Adipocyte cross-sectional area from eWAT was significantly greater in the LCR rats compared to HCR rats (**Figures 13B, 13C**). While there was no effect of the 3 d HFD on adipocyte size in either strain, heat treatment significantly decreased adipocyte size in LCR rats.

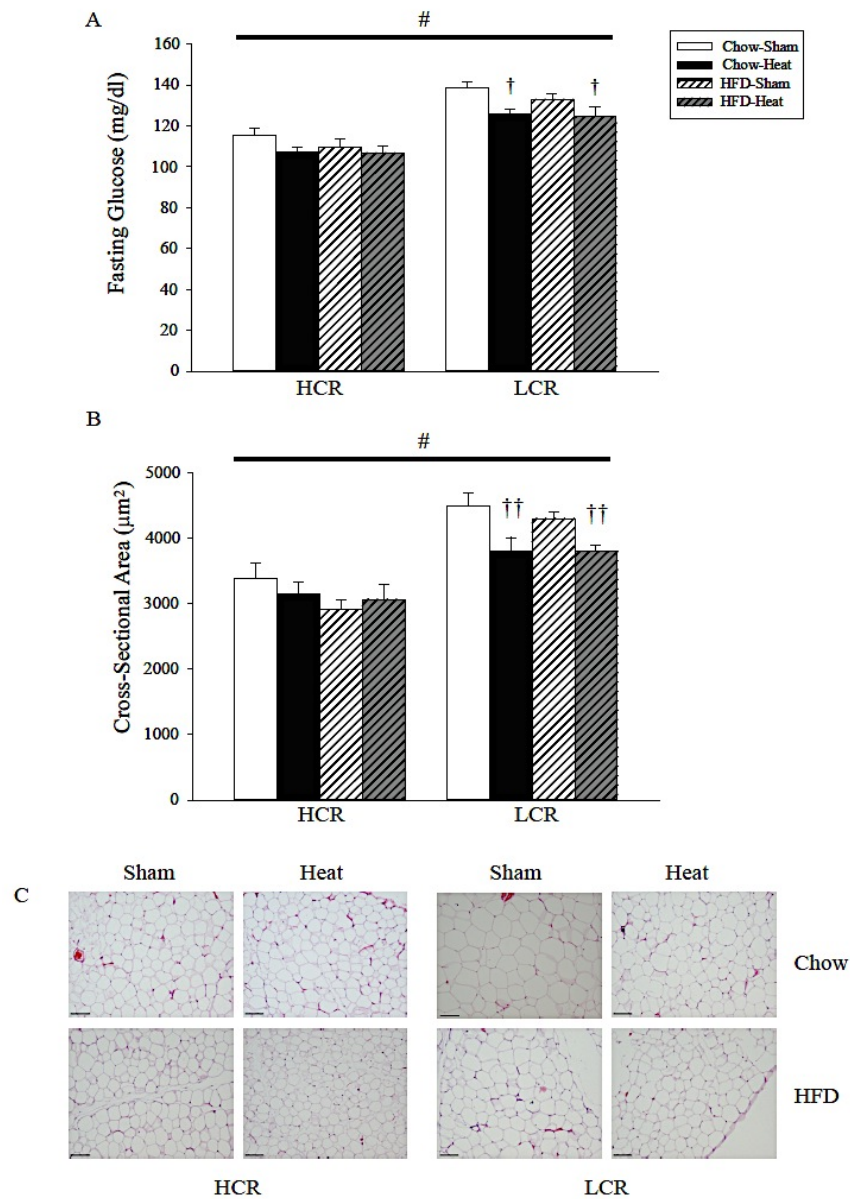


Figure 13. Blood glucose and adipocyte size following a single heat treatment. (A) Fasting blood glucose concentration and (B) cross-sectional area of adipocytes from the eWAT in HCR/LCR rat from *generation 25* fed either chow or 3 d HFD challenge and receiving a single *in vivo* sham (37°C) or heat (41°C) treatment. (C) Representative images of 10 µm thick cross-sections of eWAT. Bar represents 50 µm. # $P < 0.05$ denotes that HCR/LCR rats are significantly different within the corresponding group by one-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate. † $P < 0.05$, †† $P < 0.01$ denotes a main effect of treatment determined by two-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate in HCR/LCR rats separately. Values are means \pm SE. N = 3-6 animals per group.

In the EDL muscle, insulin-stimulated glucose uptake was not significantly different between HCR/LCR rats on the chow diet; however, basal or non-insulin-stimulated glucose uptake (open bars in **Figure 14A**) was significantly greater in HCR rats compared to LCR rats. The 3 d HFD significantly reduced insulin-stimulated glucose uptake in the EDL muscle from LCR rats, and this was restored by acute heat treatment. Similarly, insulin-stimulated phosphorylation of AS160 was significantly decreased in LCR following a 3 d HFD challenge and this was restored by heat treatment (**Figure 14C**). Insulin-stimulated Akt phosphorylation in the EDL was not significantly different between HCR/LCR rats and was not significantly changed following HFD or heat treatment (representative blots only shown, **Figure 14C**). Insulin-stimulated glucose uptake in the soleus was significantly greater in HCR rats compared to LCR rats (**Figure 14B**), but soleus muscle glucose uptake was not impacted by the 3 d HFD or heat treatment in HCR/LCR rats.

Increases in intramuscular lipid storage are intimately linked to reduced skeletal muscle insulin sensitivity (340) and therefore we examined intramuscular triglyceride content in another glycolytic muscle, the white gastrocnemius. Triglyceride content increased following a 3 d HFD in LCR rats but did not change in HCR rats (**Figure 14D**). Heat treatment effectively decreased intramuscular triglyceride content in LCR rats fed a 3 d HFD.

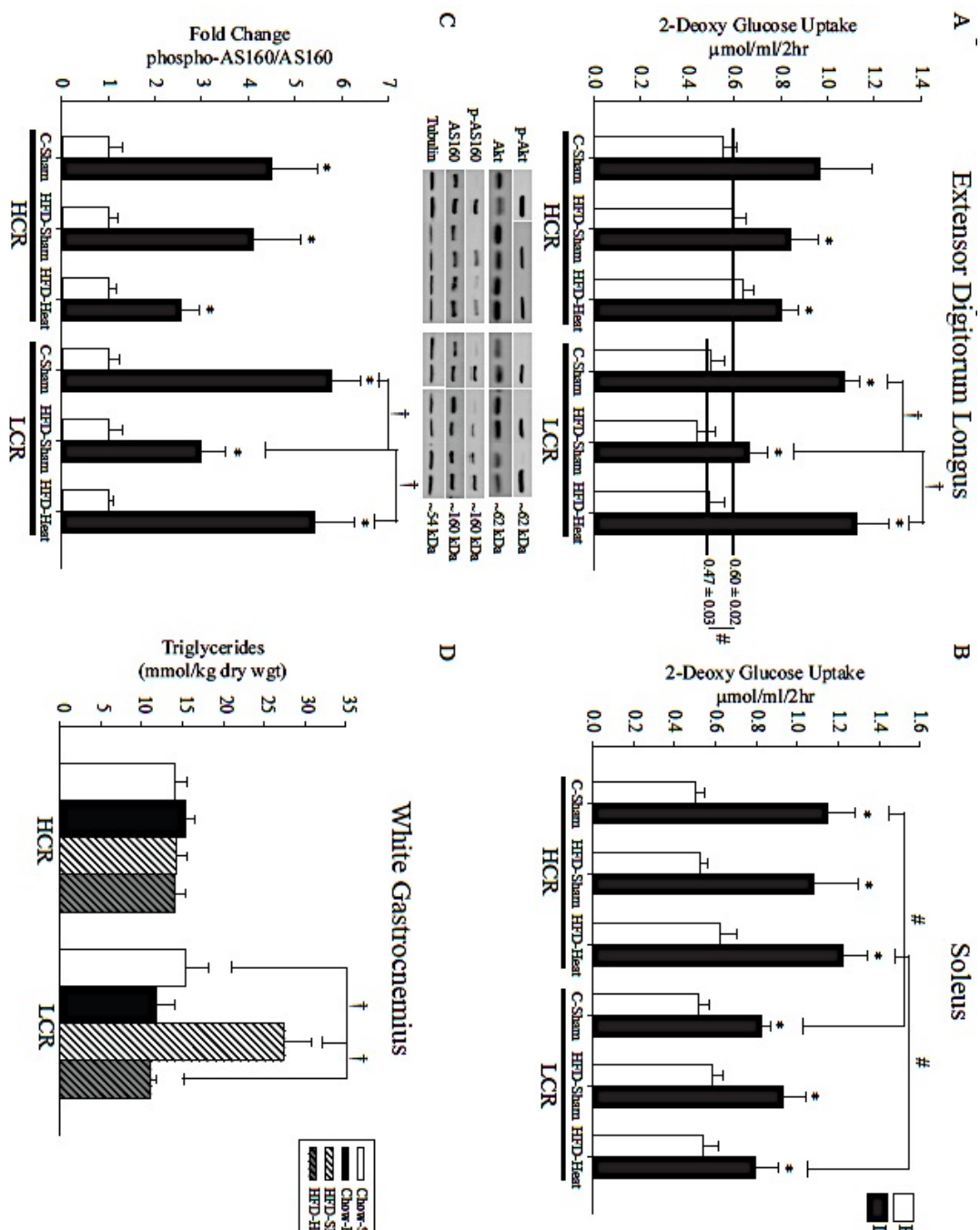


Figure 14. Skeletal muscle insulin-stimulated glucose uptake in HCR/LCR rats fed a 3 d high-fat challenge and 24 hr following a single *in vivo* heat treatment. Insulin-stimulated glucose uptake in the soleus muscle (A) and EDL muscles (B) and insulin-stimulated phosphorylation of insulin signaling proteins (C) in HCR/LCR rat from *generation 25* fed either chow (C) or 3 d HFD challenge and receiving a single *in vivo* sham (37°C) or heat (41°C) treatment. Insulin-stimulated glucose uptake was determined by incubating muscles in the presence or absence of 1 mU/ml insulin for exactly 20 min. * $P < 0.05$ -0.001 denotes a significant increase in glucose uptake determined by one-way ANOVA with Fisher's LSD post-hoc comparisons where appropriate. # $P < 0.05$ denotes that HCR/LCR rats are significantly different within the corresponding group and † denotes that LCR HFD-Sham treated rats had significantly lower insulin-stimulated glucose uptake than LCR C-Sham treated rats and LCR HFD-Heat treated rats determined by one-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate. (D) Triglyceride content of the white gastrocnemius. *, † $P < 0.05$ denotes a significant diet x treatment interaction determined by two-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate in HCR/LCR rats separately. Values are means \pm SE. N = 3-6 animals per group.

Constitutive HSP expression in glycolytic and oxidative muscles from HCR/LCR rats.

Constitutive HSP72 levels were significantly greater in the EDL of HCR rats compared to LCR rats, while constitutive HSP72 expression in the soleus muscle was not different between HCR/LCR rats (Open bars, **Figures 15A, 15B**). We found similar fiber type-specific effects in the red and white gastrocnemius muscles. Both HSF-1, the transcription factor responsible for HSP72 induction following heat stress (9), and HSP72 expression levels were significantly greater in the white gastrocnemius of HCR rats compared to LCR rats (**Figures 16A, 16B**). Similar to what was observed in the oxidative soleus muscle, no significant differences in HSF-1 and HSP72 were observed in the red gastrocnemius from HCR/LCR rats (**Figures 16D, 16E**). Constitutive levels of HSP72 were not altered by a 3 d HFD in HCR or LCR rats (**Figures 15A, 15B**).

Heat treatment resulted in an increase in HSP72 expression in the EDL muscles from HCR and LCR rats, while HSP72 expression was increased in soleus muscles from HCR rats only (**Figures 15A, 15B**). In the EDL muscle, a significant decrease in HSP72 induction occurred in LCR rats fed a HFD. HSP72 induction did not occur in soleus muscles from chow- or HFD-fed LCR rats.

Constitutive HSP25 levels were not different between HCR/LCR rats in the EDL or soleus (**Figures 15C, 15D**), or in the white or red portion of the gastrocnemius (**Figures 16C, 16F**). 24 h following heat treatment, there was no increase in total HSP25 levels in either the EDL or soleus muscle from HCR and LCR rats (**Figures 15C, 15D**). Constitutive HSP60 expression levels were significantly greater in the EDL, but not the soleus, of sham treated HCR rats compared to LCR rats (**Figures 15E, 15F**). In the EDL of LCR rats, heat treatment increased HSP60 expression in chow fed animals only (**Figure 15E**).

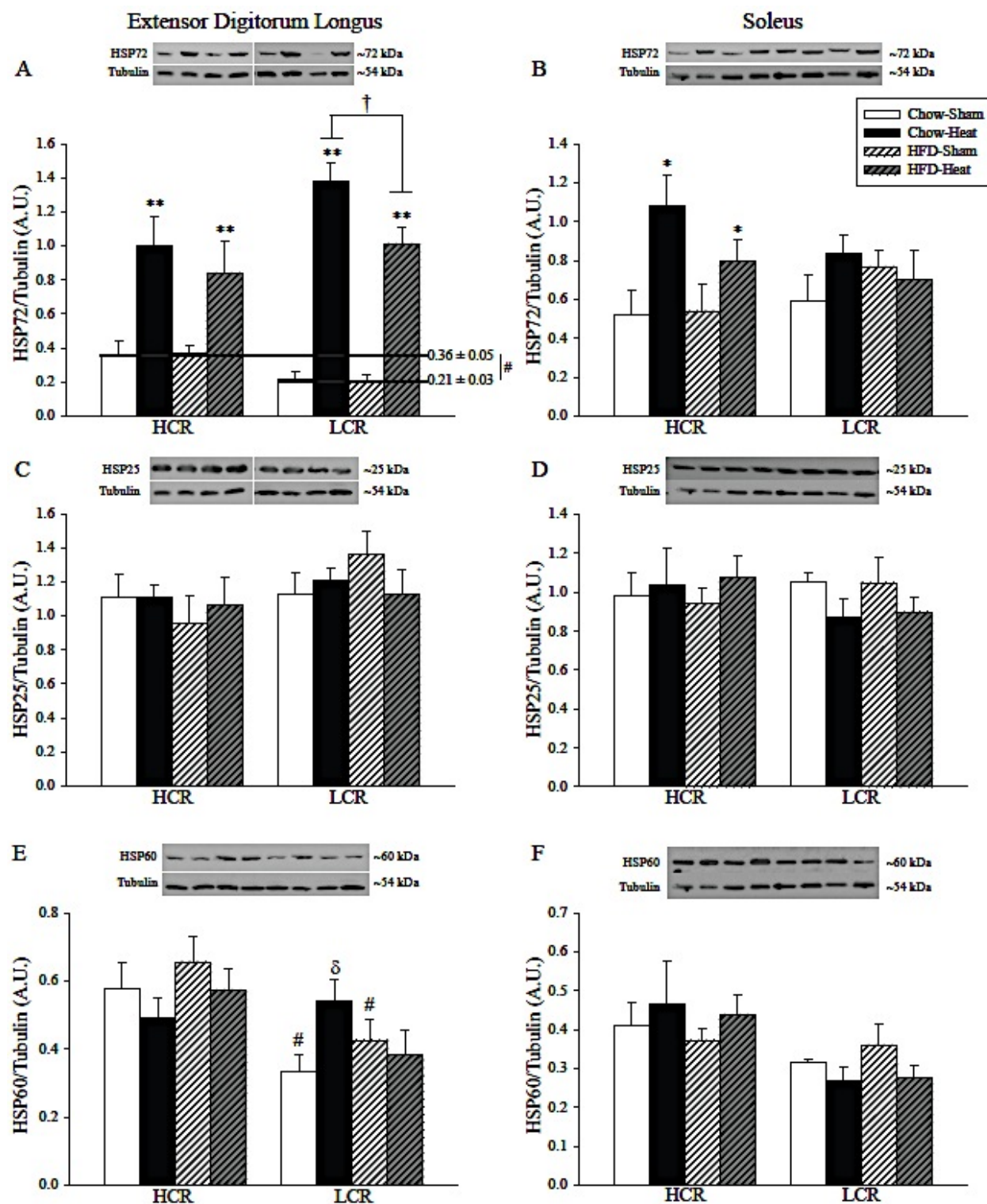


Figure 15. Induction of HSP72 and HSP25 in skeletal muscle of HCR/LCR rats. HSP72, HSP25, and HSP60 expression in the EDL (A, C, E) and soleus (B, D, F) muscles of male HCR/LCR rats from *generation 25* following heat treatment. HCR/LCR rats were fed a chow or 3 d HFD challenge and received either a single *in vivo* sham (37°C) or heat (41°C) treatment. Protein levels were normalized to α -tubulin protein levels. * $P < 0.01$, ** $P < 0.001$ denotes significant main effect of treatment, † $P < 0.05$ denotes a significant diet x treatment interaction determined by two-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate in HCR/LCR rats separately. # $P < 0.05$ denotes significantly different from HCR rats of corresponding treatment group. δ $P < 0.05$ denotes significantly different from sham by one-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate. Values are means \pm SE. N=5-6.

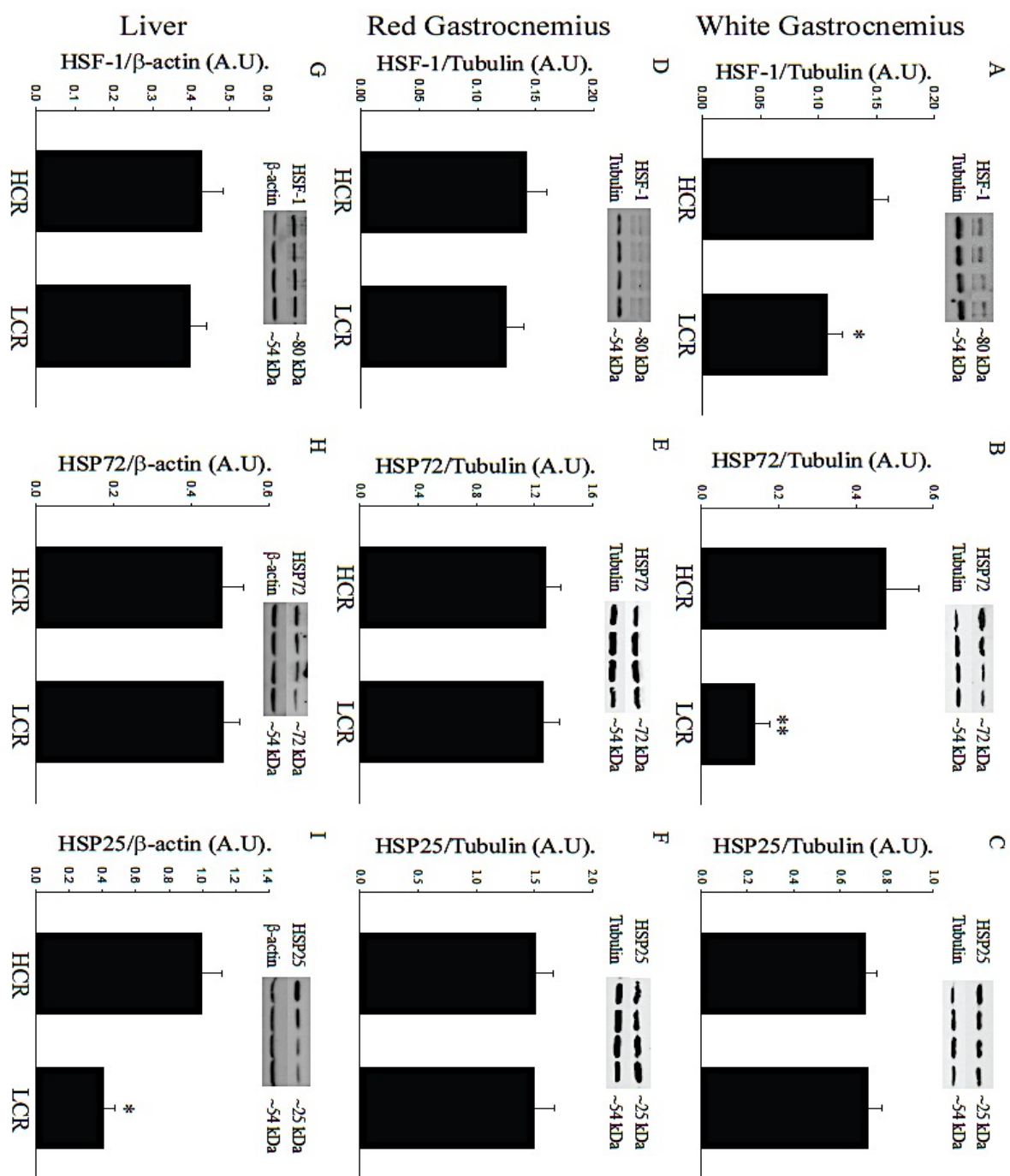


Figure 16. Constitutive HSF-1, HSP72, and HSP25 levels in skeletal muscle and liver of HCR/LCR rats. HSF-1, HSP72, and HSP25 in the white gastrocnemius (A-C), red gastrocnemius (D-F), and liver (G-I) of male HCR/LCR rats from *generation 25* or *30*. Protein levels were normalized to α -tubulin protein levels in the muscle and β -actin protein levels in the liver. * $P < 0.05$, ** $P < 0.01$ denotes significantly different determined by one-way ANOVA with Fisher's LSD post-hoc comparisons where appropriate. Values are means \pm SE. N = 12-16 samples per group.

Diet- and heat-treatment effects in the liver from HCR/LCR rats. Because previous studies have demonstrated a significant effect on the liver with a 3 d HFD (262, 427), we examined potential differences in liver HSP expression and induction in our study. Constitutive expression of liver HSP72 and HSF-1 did not differ between HCR and LCR rats (**Figures 16G, 16H**). However, HSP25 was significantly greater in liver from HCR rats compared to LCR rats (**Figure 16I**). Liver HSP72 and HSP25 were significantly increased following heat treatment in both HCR and LCR rats (**Figures 17A, 17B**), and the induction of HSP72 was significantly greater in LCR rats compared to HCR rats fed a chow diet. The induction of HSP72 and HSP25 in the liver was significantly reduced following a 3 d HFD in LCR rats (**Figures 17A, 17B**).

The stress kinase JNK is a pro-inflammatory protein that can effectively inhibit insulin signaling (5) and the primary HSP transcription factor, HSF-1 (369). Phosphorylation of JNK increased in the liver of HCR rats following heat treatment, but was not significantly changed by a 3 d HFD challenge in HCR rats (**Figure 17C**). In LCR rats, JNK phosphorylation in the liver was significantly increased by the 3 d HFD, and was significantly reduced following heat treatment in HFD-fed LCR rats. In addition, total JNK expression was significantly greater in LCR rats compared to HCR rats (**Figure 17D**), but unchanged following a 3 d HFD or heat treatment in either strain.

Liver triglyceride content was significantly greater in the LCR rats compared to HCR rats fed a chow diet (**Figure 17E**). In LCR rats, heat treatment significantly reduced liver triglyceride

content in both chow fed and high-fat fed animals (**Figure 17E**). Heat treatment did not alter liver triglyceride content in HCR rats. In addition to inhibition by stress kinases, HSF-1 is inactivated by acetylation (521). To determine whether decreased HSP induction in the liver with a HFD could be due to acetylation of HSF-1, we examined acetylation of lysine residues in liver homogenates from LCR rats. The acetylation pattern was greater in LCR rats fed a 3 d HFD and was reduced by heat treatment (**Figure 17F**).

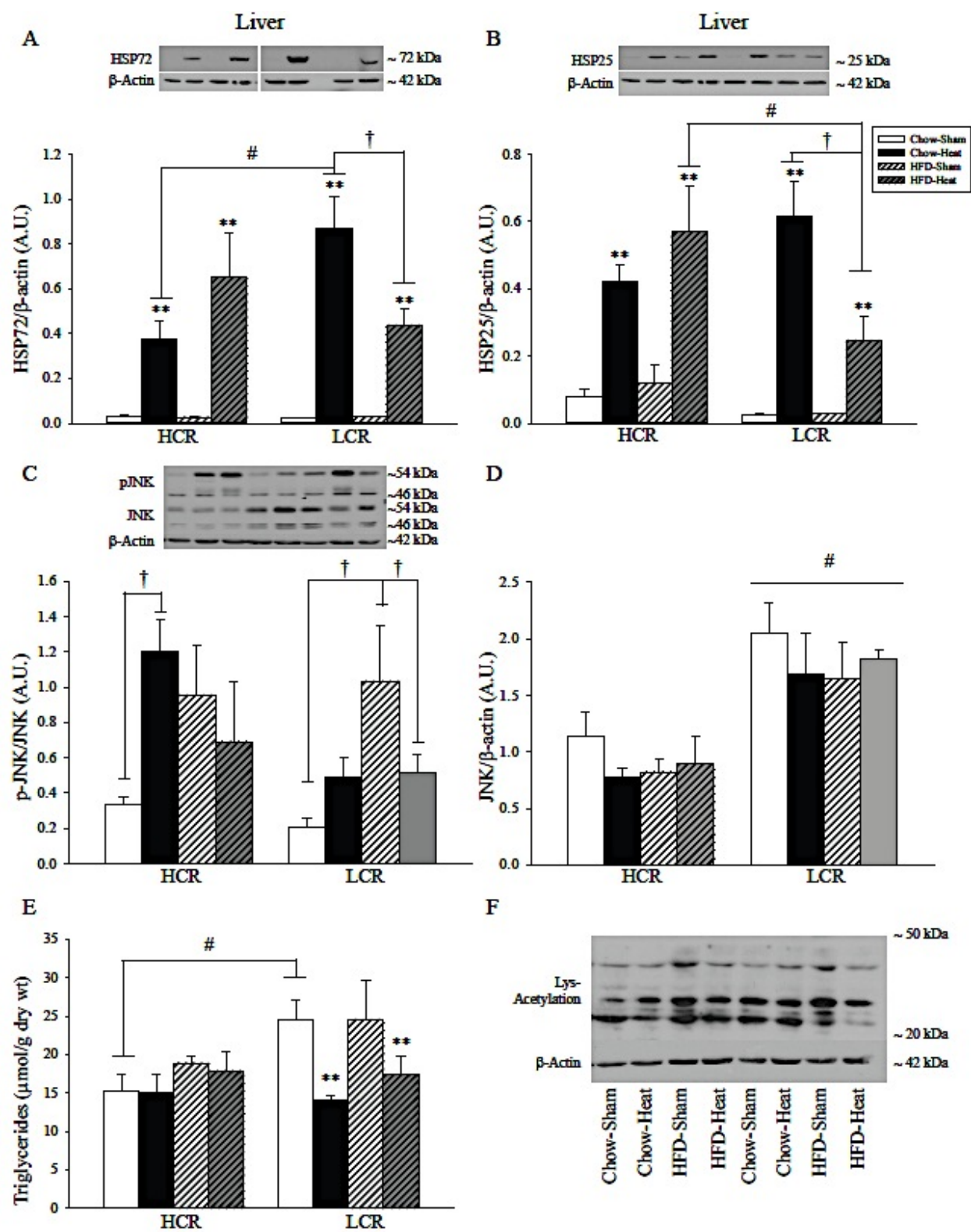


Figure 17. HSP response and JNK phosphorylation and total levels in liver of HCR/LCR rats fed a 3 d high-fat challenge and following a single *in vivo* heat treatment. (A) HSP72, (B) HSP25, (C) phosphorylation of JNK, (D) total JNK levels, and (E) triglyceride content in liver of male HCR/LCR rats from *generation 25*. (F) Acetylation of lysine residues in liver homogenates of HCR/LCR rats. Rats were fed a chow or 3 d HFD challenge and received either a single *in vivo* sham (37°C) or heat (41°C) treatment. Phosphorylation of JNK levels were normalized to total JNK levels and levels of other proteins were normalized to β -actin protein levels. ** $P < 0.01$ denotes a significant main effect of treatment, † $P < 0.05$ denotes a significant diet x treatment interaction determined by two-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate in HCR/LCR rats separately. # $P < 0.05$ denotes significantly different from HCR rats of corresponding treatment group. Values are means \pm SE. N=5-6.

Mitochondrial protein levels are associated with HSPs in skeletal muscle of HCR/LCR rats. In the white gastrocnemius muscle, a mitochondrial HSP, HSP60, was greater in HCR rats compared to LCR rats. A similar pattern was observed for mitochondrial respiratory chain complexes I (NADH dehydrogenase ubiquinone 1 beta subcomplex 8, NDUFB8), II (succinate dehydrogenase subunit B, SDHB), III (ubiquinol cytochrome c reductase core protein 2, UQCRC2), and IV (cytochrome c oxidase subunit I, MTCO1) (**Figures 18A-E**). In addition, PGC-1 α , mitochondrial transcription factor A (mtFAM), and sirtuin 1 (Sirt1) levels were significantly greater in HCR rats compared to LCR rats (**Figures 18G-I**). These differences observed in HSP60 levels, content of mitochondrial complexes, and PGC-1 α was similar in the white quadriceps (data not shown).

In the red gastrocnemius muscle, HSP60 levels and content of mitochondrial respiratory chain complexes I, III, IV, as well as mtFTAM were significantly greater in HCR rats compared to LCR rats (**Figures 19A, 19B, 19D, 19E, and 19H**), but content of complexes II and V, and PGC-1 α and Sirt1 levels were not significantly different between strains (**Figures 19C, 19F, 19G, and 19I**). These differences observed in HSP60 levels, content of mitochondrial complexes, and PGC-1 α was similar in the red quadriceps (data not shown). The magnitude of

the differences in the content of respiratory chain complexes between the HCR and LCR rats was greater in the white portion of the gastrocnemius compared to the red portion. Complex I expression was 5.6-fold greater, complex II 1.7-fold greater, complex III 5.8-fold greater, and complex IV 3.2-fold greater in the white gastrocnemius of HCR rats compared to LCR rats, but was 1.9-fold, 1.1-fold, 1.7-fold, and 2.7-fold greater, respectively, in the red gastrocnemius of HCR rats compared to LCR rats. HSP72 expression was significantly correlated with levels of complex I ($R = 0.515$, $P = 0.003$), complex III ($R = 0.482$, $P = 0.005$), complex IV ($R = 0.517$, $P = 0.002$), PGC-1 α ($R = 0.499$, $P = 0.004$), and a trend with mtFAM ($R = 0.347$, $P = 0.051$) (**Figure 20A, 20C, 20D, 20F, 20G**). Similarly, HSP60 expression was significantly correlated with levels of complex I ($R = 0.422$, $P = 0.011$), complex III ($R = 0.387$, $P = 0.028$), complex IV ($R = 0.386$, $P = 0.028$), PGC-1 α ($R = 0.392$, $P = 0.027$), and mtFAM ($R = 0.476$, $P = 0.006$) (**Figure 20H, 20J, 20K, 20M, 20N**).

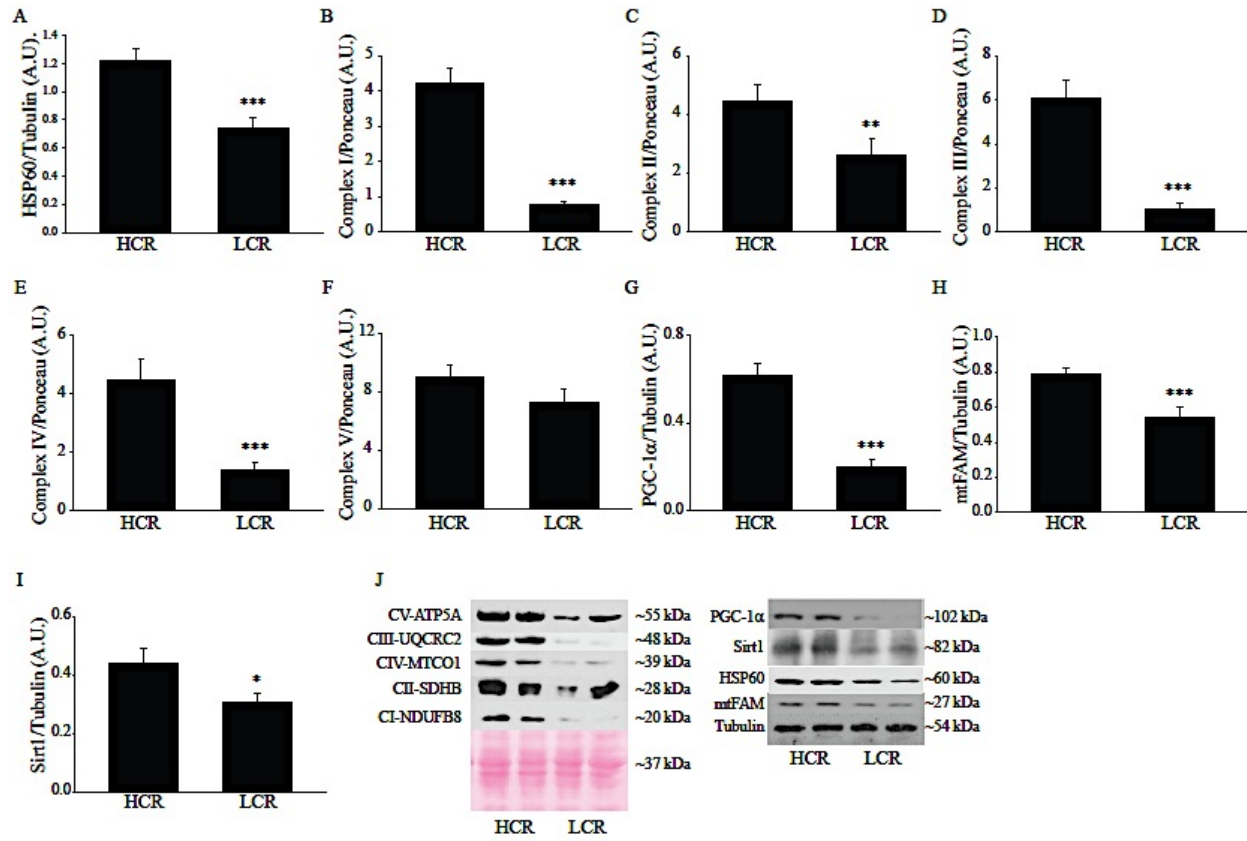


Figure 18. Mitochondrial protein expression in white gastrocnemius muscle of HCR/LCR rats. Mitochondrial protein expression of HSP60 (A), complex I (B), complex II (C), complex III (D), complex IV (E), complex V (F), PGC-1 α (G), mtFAM (H), Sirt1 (I), and representative blots (J) in the white gastrocnemius muscle of male HCR/LCR rats from *generation 30*. Complex I-V protein expression was normalized to Ponceau staining. PGC-1 α , mtFAM, and Sirt1 protein levels were normalized to α -tubulin protein levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denotes that protein levels are significantly different between HCR and LCR rats determined by one-way ANOVA with Fisher's LSD post-hoc comparisons performed where appropriate. Values are means \pm SE. N = 12-16 samples per group.

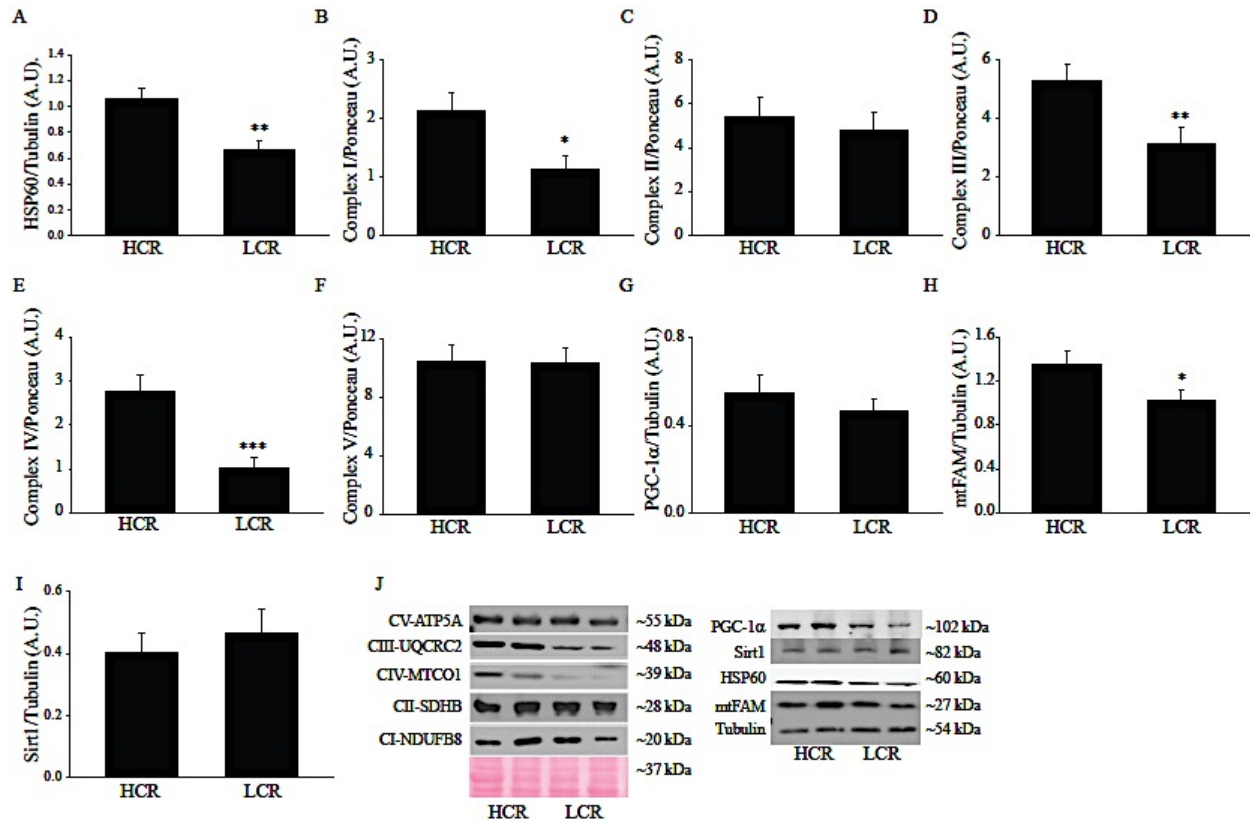


Figure 19. Mitochondrial protein expression in red gastrocnemius muscle of HCR/LCR rats. Mitochondrial protein expression of HSP60 (A), complex I (B), complex II (C), complex III (D), complex IV (E), complex V (F), PGC-1α (G), mtFAM (H), Sirt1 (I), and representative blots (J) in the red gastrocnemius muscle of male HCR/LCR rats from *generation 30*. Complex I-V protein expression was normalized to Ponceau staining. PGC-1α, mtFAM, and Sirt1 protein levels were normalized to α-tubulin protein levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denotes that protein levels are significantly different between HCR and LCR rats determined by one-way ANOVA with Fisher's LSD post-hoc comparisons where appropriate. Values are means \pm SE. $N = 14-16$ samples per group.

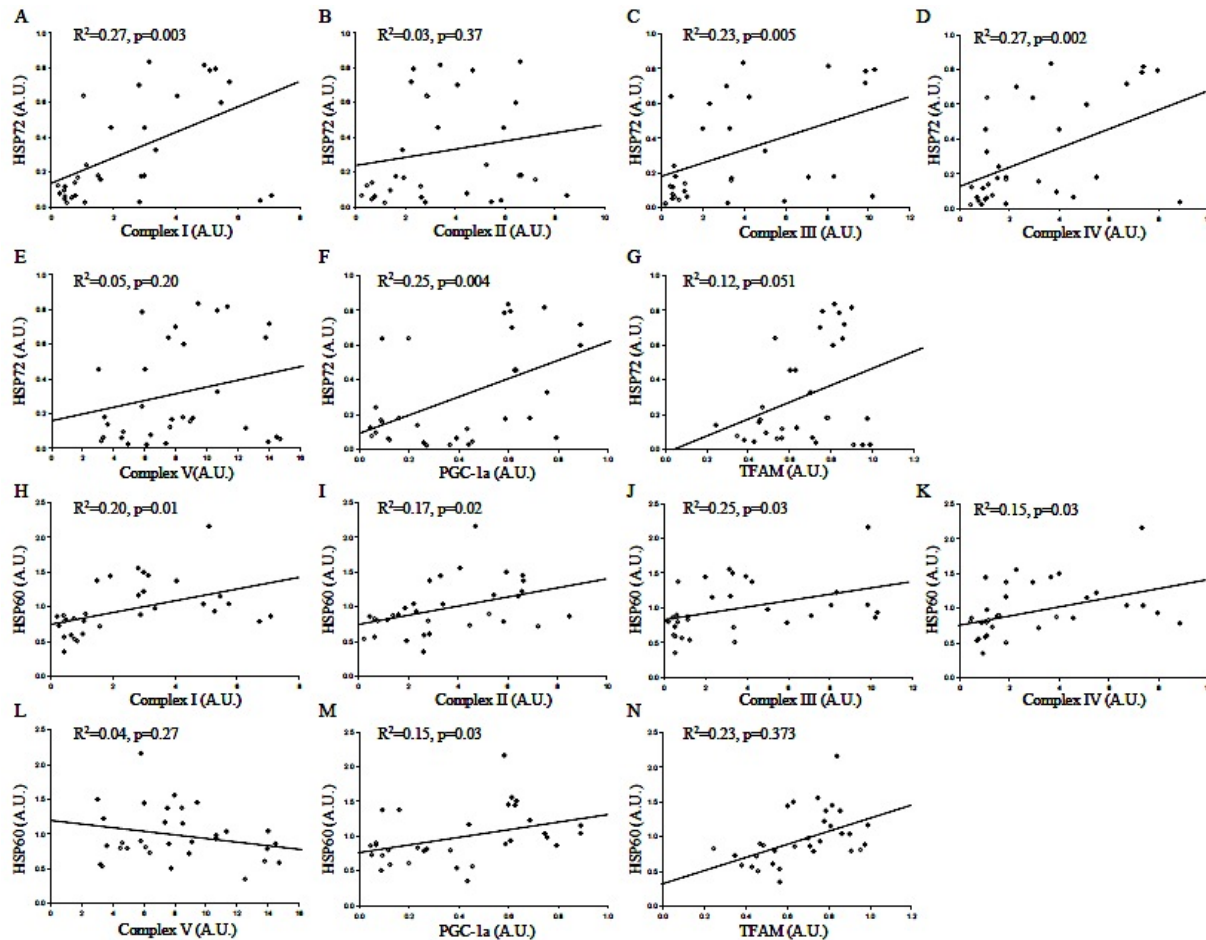


Figure 20. Correlations between HSP72, HSP60, and mitochondrial proteins in white gastrocnemius of HCR/LCR rats. Correlations between HSP72 and HSP60 and respiratory chain complex I (A, H), complex II (B, I), complex III (C, J), complex IV (D, K), complex V (E, L), PGC-1α (F, M), and mtFAM (G, N). Muscles from male HCR/LCR rats from *generation 30* were used. Protein levels were measured by Western blotting and protein levels normalized to α -tubulin protein levels. Correlations were determined by linear regression. Open circles represent LCR rats and closed circles represent HCR rats. N = 14-16 samples per group.

Differences in autophagy and mitophagy in HCR/LCR rats following a 3 d HFD. Recent evidence suggests that autophagy and/or mitophagy (the breakdown of mitochondria by autophagic processes) (99) is regulated by HSP72 and a loss of HSP72 results in dysregulated autophagy/mitophagy (101, 173). LC3 is lipidated from LC3-I forming LC3-II, which is considered a marker of activated autophagy. LC3-II levels were not significantly different between HCR/LCR rats in the white gastrocnemius (**Figure 21A**). LC3-II levels significantly increased in white gastrocnemius from HCR rats fed a 3 d HFD compared to chow fed, but this response did not occur in the LCR rats in response to the HFD (**Figure 21A**). Results were similar when LC3-II was expressed relative to LC3-I (data not shown).

LC3-II levels were significantly greater in the red gastrocnemius of HCR rats compared to LCR rats (**Figure 21B**). Similar results were found when LC3-II levels were expressed relative to LC3-I (data not shown). There was a trend for LC3-II levels to increase following a 3 d HFD in the red gastrocnemius of HCR and LCR rats (main effect of diet); however, when compared by one-way ANOVA, there was not a significant increase in LC3-II levels in HCR rats or LCR rats fed a 3 d HFD. LC3-I levels were not significantly different between HCR/LCR rats, and not changed following a 3 d HFD challenge in the white or red gastrocnemius (**Figure 21C, 21D**). Parkin is a protein essential in the regulation of mitophagy and Parkin levels were significantly greater in the white gastrocnemius of the LCR rats compared to HCR rats (**Figure 21E**). In the red gastrocnemius, Parkin levels were significantly greater in the LCR rats compared HCR rats (**Figure 21F**). Parkin levels significantly increased in LCR following a 3 d HFD challenge, accounting for these strain differences (**Figure 21F**). Parkin levels did not change in the red gastrocnemius of HCR rats following a 3 d HFD.

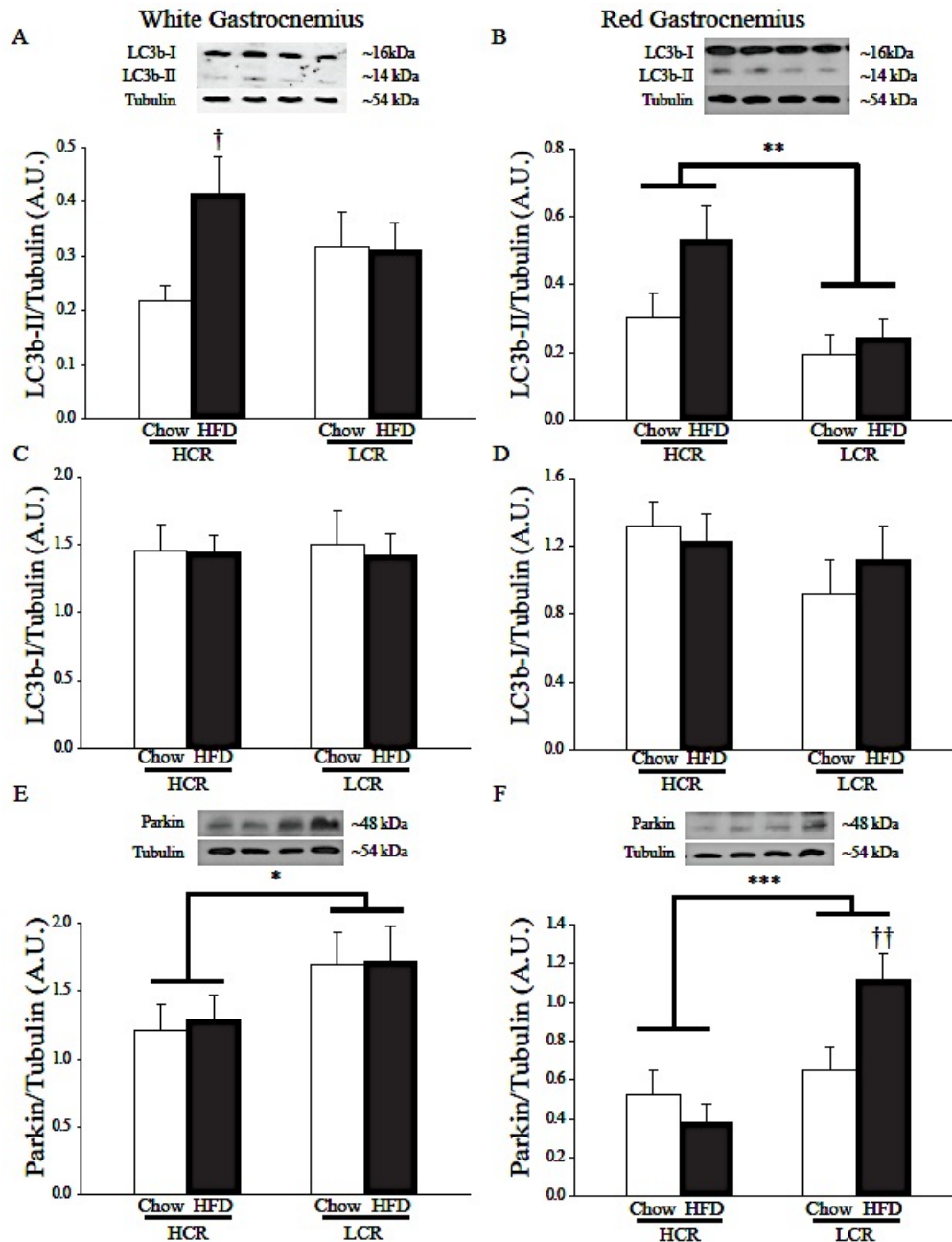


Figure 21. Autophagy and mitophagy proteins following 3 d HFD challenge in HCR and LCR rats. LC3-II in white (A) and red (B) portions of the gastrocnemius, LC3-I levels in white (C) and red (B) gastrocnemius, and Parkin levels in white (E) and red (F) gastrocnemius of male HCR and LCR rats from *generation 30* fed a chow or 3 d HFD challenge. Protein levels were normalized to α -tubulin protein levels. Similar results were observed when LC3-II was expressed relative to LC3-I. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denotes a significant main effect of strain, and $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ denotes a significant diet \times strain interaction determined by two-way ANOVA with Fisher's LSD post-hoc comparisons where appropriate. Values are means \pm SE. N = 6-8 samples per group.

4.5. DISCUSSION

New findings from the present study demonstrate the ability of an acute heat treatment to mitigate metabolic dysfunction following a 3 d HFD challenge. In a model of low intrinsic aerobic capacity, the glycolytic skeletal muscle is particularly susceptible to an acute HFD. LCR rats develop skeletal muscle insulin resistance and increase triglyceride stores in glycolytic muscles only, while both oxidative and glycolytic muscle from HCR rats are largely protected. Previous studies have demonstrated an early effect of a 3 d HFD on the liver; however, our findings are the first to demonstrate metabolic defects in skeletal muscle as a result of a 3 d HFD. The mechanisms by which low aerobic capacity lead to increased susceptibility to metabolic disease, and by which high aerobic capacity plays a protective role have not been elucidated. Our findings suggest that lower constitutive levels of HSP72, HSF-1, HSP60, as well as mitochondrial proteins that determine oxidative capacity, may underlie the increased susceptibility of LCR rats to an acute metabolic insult. Beyond muscle, adipocyte size and triglyceride storage in the liver were also decreased with a single heat treatment demonstrating beneficial metabolic adaptations in multiple tissues.

Previously, Morris *et al.* reported that LCR rats display metabolic inflexibility when fed an acute dietary challenge (3 d high-fat feeding) compared to HCR rats, results that were associated with the development of hepatic steatosis in LCR rats (332). Here we expand upon this concept by showing that when fed a 3 d HFD, LCR rats suffer a decrement in insulin sensitivity primarily in glycolytic muscle. A single heat treatment was able to improve glucose homeostasis, restore insulin sensitivity, and reduce triglyceride content in in glycolytic skeletal muscle of LCR rats. We did not observe differences in glycolytic muscle insulin sensitivity in chow fed HCR/ LCR rats. However, Rivas *et al.* reported that phosphorylation of insulin

signaling proteins and total levels of Akt1 and Akt2 were lower and AS160 levels higher in glycolytic muscle of LCR rats compared to HCR rats, but when phosphorylation of these proteins was normalized to total protein levels there were no differences between HCR/LCR rats indicating that insulin responsiveness may not be different between HCR/LCR rats in the glycolytic muscle (407). Differences in the sex of the animals, as well as differences in the models used to test skeletal muscle insulin sensitivity may explain these different results.

Deposition of dietary lipids into skeletal muscle following a 3 d HFD challenge was greater in the HCR rats compared to LCR rats (332), which is supported by data from others showing that HCR rats have greater content of plasma membrane fatty acid transport protein compared to LCR rats (285). In the present study, we observed that triglyceride content appreciably increased in the glycolytic muscle of LCR rats following a 3 d HFD challenge despite lower capacity to uptake fatty acids shown elsewhere (332, 407). This increased triglyceride content may be a result of defective mitochondrial lipid handling. LCR rats have been shown to have lower mitochondrial content and functionality in the skeletal muscle compared to HCR rats (286, 355, 407, 442, 525), and in the present study we observed that differences between HCR/LCR rats in the content of the respiratory chain complexes were most pronounced in the glycolytic muscle. This is supported by findings from Rivas *et al.* (407) showing differences in mitochondrial enzyme activity are more pronounced between LCR/HCR rats in glycolytic muscle compared to oxidative muscle. Thus, HCR rats appear to have the ability to preferentially import and oxidize fatty acids when provided a 3 d HFD challenge, a sign of enhanced metabolic flexibility. The improvements in insulin sensitivity and triglyceride content in glycolytic muscle of LCR rats following heat treatment may represent improvements in mitochondrial function discussed in more detail below.

Previous studies have demonstrated an important association between expression levels of HSP72 and metabolic disease showing that constitutive levels of HSP72 and HSF-1 are lower in skeletal muscle of type 2 diabetics and diabetic rodents (17, 50, 67, 269, 375, 411). As a model of low intrinsic aerobic capacity, LCR rats display symptomology of metabolic syndrome with reduced whole-body and skeletal muscle insulin sensitivity shown here and elsewhere (286, 332, 355, 357, 407, 476, 525). This is the first study to fully characterize constitutive expression of HSPs in skeletal muscle of HCR and LCR rats. HSP72 was significantly higher in HCR rats compared to LCR rats, but only in glycolytic skeletal muscles (EDL, white gastrocnemius). Previously we have shown that HSP72 protein expression is associated with oxidative capacity and expression levels are highest in slow-twitch muscles like the soleus, compared with glycolytic muscles like the EDL (152). It may therefore be a positive adaptation of the HCR rats that they have increased HSP72 expression in glycolytic muscles, which served to protect the muscles from a 3 d HFD in the present study.

The mechanisms by which HSP72 protects against insulin resistance are not completely understood, but are likely to be multifactorial. Recently, HSP72 has been shown to be a powerful regulator of mitochondrial function and the processes of autophagy/mitophagy. Overexpression of HSP72 in skeletal muscle results in a near 50% increase in mitochondrial content and an approximate 2-fold increase in endurance running capacity (173). In contrast, mice with global knockout of HSP72 display whole-body and skeletal muscle insulin resistance, as well as deficits in fatty acid oxidation, and more recently Drew *et al.* established that HSP72 is essential for proper function of mitophagy – the autophagic process by which damaged mitochondria are cleared (99, 101). Loss of HSP72 results in an increase in Parkin levels, an E3 ubiquitin ligase thought to be essential for mitophagy (99); however, the available Parkin is unable to translocate

to the mitochondria properly and target dysfunctional mitochondria for degradation through mitophagy (101). In the present study, muscles from LCR rats with reduced HSP72 levels also had increased Parkin levels similar to that observed in HSP72 knockout mice. In the oxidative soleus muscle, Parkin levels were not different between HCR/LCR rats. Further, Parkin levels increased in LCR rats following a 3 d HFD that suggests that LCR rats may be attempting to clear dysfunctional mitochondria.

The induction of HSPs and HSF-1 DNA binding are blunted in insulin resistant or diabetic animals (17, 236). In the present study, we show that the induction of HSP72 following heat treatment was different between HCR/LCR rats in oxidative muscle, but similar in glycolytic muscle despite lower HSF-1 levels in glycolytic muscle in LCR rats. The induction of HSP72 was actually greater in the liver of LCR rats compared to HCR rats following heat treatment. Additionally, HSP72 induction was blunted in the EDL and liver of LCR rats following a 3 d HFD challenge and maintained in HCR rats. The regulation of constitutive HSP levels by HSF-1 under unstressed conditions has not been resolved (64), and the partial loss of HSF-1 results in blunted induction of HSP levels following heat stress but not a complete abolishment of constitutive HSP expression (211, 312, 543). Thus, other mechanisms must be regulating constitutive HSP72 as well as its induction rather than simply constitutive HSF-1 levels.

High-fat feeding has also been shown to increase acetylation of lysine residues in skeletal muscle and liver (339), and HSF-1 acetylation reduces its DNA binding activity. Sirt1 is a deacetylase that has been shown to directly deacetylate HSF-1 allowing for longer DNA binding (521). Previous studies have shown a decrease in skeletal muscle Sirt1 levels and an increase in acetylation of mitochondrial proteins in LCR rats (357, 362). In the present study, we observed

lower Sirt1 levels in LCR rats only in the white gastrocnemius where HSF-1, HSP72 and HSP60 are also decreased. In addition to acetylation, HSF-1 can be inhibited by phosphorylation by stress kinases such as GSK3, ERK, and JNK (65, 66, 369). In the current study, JNK phosphorylation in the liver was significantly increased by a 3 d HFD and reduced by heat treatment in LCR rats that has been shown elsewhere by heat treatment or HSP72 induction (3, 330). This may represent an improved inflammatory profile in LCR rats following heat treatment. Further investigation of the ability of chronic heat treatment to protect against hepatic steatosis is warranted.

CONCLUSION

We demonstrate that a unique model of low aerobic capacity is susceptible to insulin resistance in glycolytic muscle with a short term high-fat diet, a trend that parallels reduced skeletal muscle content of HSP72, HSF-1, and mitochondrial proteins. In contrast, HCR display elevations in HSP72, HSF-1 and mitochondrial proteins and display protection against acute high fat diet induced changes. Heat exposure improves the phenotype of the LCR while increasing HSP72 protein. This research further illustrates that intrinsic aerobic capacity plays a large role in whole body susceptibility to metabolic diseases, and that heat shock protein expression may play an important role in these processes.

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DISCLOSURES

The authors have no conflicts of interest, financial or otherwise, to disclose for this research.

CHAPTER 5

SUMMARY OF RESULTS, DISCUSSION, AND FUTURE DIRECTIONS

Heat treatments and the induction of HSP72 have been shown, and confirmed here, to improve whole-body glucose homeostasis and skeletal muscle insulin sensitivity. With the present studies, we expand the metabolic role of HSP72 into other tissues, including the WAT and liver. We also implicate decreased HSP levels as one factor influencing susceptibility to high-fat feeding in a model of low CRF. These experiments strengthen a growing body of work connecting HSP72 and mitochondrial function with autophagy-mitophagy.

5.1. HEAT SHOCK PROTEINS IN THE ADIPOSE TISSUE

It is largely unknown what role the HSPs may play in WAT metabolism or the dysfunction of WAT during obesity and insulin resistance. Obesity in humans has been shown to increase HSP levels in the WAT, although HSP levels are lower in WAT of obese type 2 diabetics (97, 382, 383, 478). Tiss *et al.* observed that HSP72 is increased in WAT of obese humans, but the HSP72 co-chaperone, DnaJ/HSP40, was down regulated, which may reduce the functional capacity of HSP72 (478). The mechanisms by which these changes in HSP and co-chaperone levels in the WAT occur during obesity need further investigation. An attractive hypothesis is that increased levels of ROS that occur during obesity and insulin resistance introduce added stress upon the cellular environment leading to increased HSP levels. However, once metabolic derangements become too great, HSP levels fall off possibly due to the down regulation of HSF-1 or inhibition of HSF-1 by stress kinases (ie., JNK, GSK), which occurs in skeletal muscle of type 2 diabetics (411). Future investigations need to show whether induction of HSP72 or HSP25 is protective against WAT dysfunction and insulin resistance. In addition to

heat treatment to induce HSPs, transgenic overexpression and ablation experiments specific to the WAT would be insightful in elucidating the role of HSPs in WAT.

The abdominal WAT is more metabolically active than the SCAT with greater rates of lipolysis, cytokine release, and mitochondrial content (207). We observed that constitutive levels of HSP72 and HSP25 are greater in more metabolically active WAT depot, which has been supported by other laboratories (383, 421, 458), and the induction of HSP72 was greatest in the SCAT where constitutive levels are lowest. This is similar to skeletal muscle where constitutive HSP levels are higher in oxidative skeletal muscle fibers than glycolytic muscle fibers (45, 152, 278, 296, 361), and the induction of HSP levels is greatest in glycolytic muscle where constitutive levels are lowest. Greater metabolic activity of the adipocytes in the abdominal WAT may result in elevated generation of ROS leading to greater constitutive HSP levels to protect against the deleterious effects of excessive ROS.

The molecular mechanisms by which adipocytes from different WAT depots sense temperature needs further investigation and may provide insight into the inherent differences between WAT depots. Typically, heat stress is thought to induce protein unfolding, aggregation, and ER stress resulting in HSF-1 activation, and subsequently increased HSP levels, although many other stimuli that would not cause protein unfolding have been shown to increase HSP levels (329, 450). Bruce Spiegelman's laboratory has shown that the SCAT of mice is more sensitive to cold exposure than eWAT (535), and the regulation of the transient receptor potential cation channel subfamily V member 4 (TRPV4) channels likely play a role. In addition, heat stress has been shown to modulate membrane fluidity (483, 484, 490), which may also play a role in induction of HSPs. Differences in the properties of the plasma membrane and lipid droplet membrane of adipocytes from different WAT depots may explain differences in

temperature sensing between WAT depots. Finally, HSF-1 levels may be different between WAT depots, which have not been investigated to our knowledge.

5.2. METABOLIC INFLUENCE OF HEAT TREATMENT

Heat treatments may induce changes acutely (e.g., 0-6 hr following heat treatment) or chronically (> 48 hr). During our single heat treatment experiments, we monitored changes in outcomes and HSP levels 24 hr following heat treatment. For instance, WAT fatty acid handling was altered in a depot specific fashion. Heat treatment induces HSP expression but also induces a classical physiological stress response. Thus, heat treatment itself may change the hormonal milieu leading to activation of cellular enzymes involved in fatty acid metabolism and handling, although at 24 hr following heat treatment we did not observe changes in enzyme levels. It is possible that the changes observed at 24 hr, or chronically (> 48 hr following heat treatment), partially results from acute changes induced by heat treatment. For instance, in C2C12 myotubes Liu & Brooks observed that AMPK phosphorylation was increased 2 hr following heat stress leading to changes in Sirt1 and PGC-1 α expression 24 hr later ultimately resulting in increased content of respiratory chain complexes after repeated heat stress (293).

We have begun to test the acute influence of heat treatment on WAT metabolism by measuring AMPK activation 2 hr following heat stress. Male Wistar rats were heat or sham treated and WAT depots removed 2 hr following treatment. Surprisingly, HSP72 was significantly increased in the eWAT (**Figure 22A**) and SCAT (**Figure 22C**) of heat treated rats, and there was a trend for HSP72 to be increased in the rpWAT ($P = 0.09$) (**Figure 22B**). AMPK phosphorylation was significantly increased in the eWAT (**Figure 22D**) and rpWAT (**Figure 22E**).

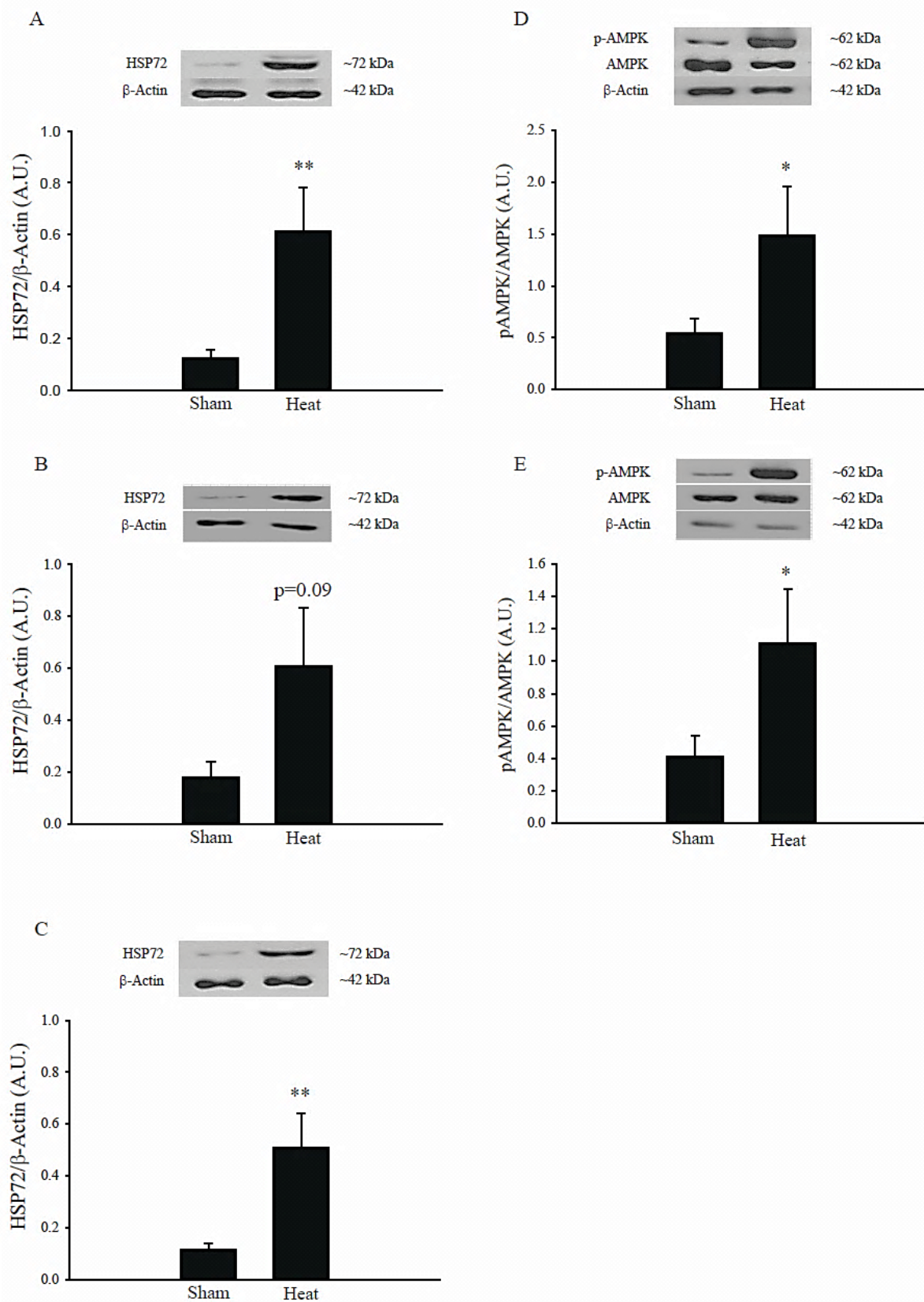


Figure 22. HSP72 expression and phosphorylation of AMPK in WAT 2 hr following a single *in vivo* heat treatment. HSP72 expression in the eWAT (A), rpWAT (B), and SCAT (C) and phosphorylation of AMPK (Thr-172) in the eWAT (D) and rpWAT (E) 2 hr following a single *in vivo* heat treatment. Male Wistar rats, 2-3 mo of age, received either a single sham (37°C, 20 min) or heat (41°C, 20 min) treatment and 2 hr following WAT was harvested and frozen at -80°C for Western blotting. * $P < 0.05$, ** $P < 0.01$ denotes a significant increase following heat treatment determined by ANOVA. Values are means \pm S.E.M. $N = 5-6$ samples per group.

AMPK's role in WAT metabolism is somewhat complex. Initially thought to be anti-lipolytic (130), AMPK is activated as a result of lipolysis and fatty acid re-esterification (131), which are energy consuming pathways. Heat treatment may induce the release of fatty acids from the WAT to fuel the recovery of other tissues, and AMPK then feeds back to inhibit lipolysis during recovery from heat treatment. Similarly, AMPK phosphorylation increases in skeletal muscle transiently following acute exercise (163, 183). This increase in AMPK activation may modulate fatty acid metabolism in response to heat treatment, and be an early step in mitochondrial biogenesis. To test the timing of AMPK activation following heat treatment, it will likely be necessary to move to an *in vitro* model. In this work, we pioneered the use of ATOCs to test for changes in protein expression following heat treatment, and this method has been used to test for changes in lipolysis as well as changes following incubation with different compounds (i.e., IL-6) (124) (468, 510, 512). Previously, our laboratory has used the compound KNK to inhibit the induction of HSPs following heat treatment (154, 155), and this compound could be used in the ATOC model to test for changes in fatty acid metabolism more mechanistically in conjunction with activation and inhibition of AMPK.

5.3 THE ROLE OF HSPS IN PREVENTING OR TREATING INSULIN RESISTANCE

In humans, CRF is largely genetically determined (46), but inactivity strongly contributes to low CRF. Low CRF aggregates in individuals with metabolic syndrome and type 2 diabetes. HSP72 expression is lower in the skeletal muscle, adipose tissue, and liver of obese, type 2 diabetics and negatively correlates with insulin sensitivity and disease progression (50, 67, 97, 174, 269, 382). The question of whether lower HSP72 levels cause insulin resistance, or are simply associated with insulin resistance has recently been addressed. Whole-body ablation of HSP72 in mice results in an obese, insulin resistant phenotype (101). This establishes a direct mechanistic link between reduced HSP72 levels and insulin resistance. However, it would be necessary to expose HSP72 knockout mice to heat treatment to verify that previously observed improvements in insulin sensitivity are absent.

In the present investigations, we observed that in LCR rats, a model of low CRF, where mitochondrial content and functionality are low, constitutive HSF-1, HSP72, and HSP60 levels are lower in glycolytic skeletal muscle compared to HCR rats. The glycolytic muscle was particularly susceptible to insulin resistance induced by a 3 d HFD challenge. The differences in HSP levels may represent another mechanism by which mitochondrial function is compromised in LCR rats, to be discussed in more detail below. We had initially believed that reduced levels of HSP60 were reflective of lower mitochondrial content in the skeletal muscle of LCR rats, but HSP60 may have a more direct role in the development of insulin resistance than initially thought. Mice with heterozygous deletion of HSP60 are insulin resistant, and hypothalamic neurons with HSP60 knockdown have reduced mitochondrial capacity, insulin resistance, and

increased inflammation (63, 247). We hope to follow-up on these observations by investigating whether HSP60 knockdown via siRNA or lentivirus in L6 myotubes results in reduced insulin-stimulated glucose uptake, which would confirm that HSP60 has a direct role in skeletal muscle insulin responsiveness.

It should be noted that LCR rats have the ability to improve their metabolic derangements following exercise training (286, 460). Most humans with low CRF can also improve their metabolic derangements through increased physical activity and exercise although there is a great deal of individual differences in the response and reports of non-responders. Similarly, heat treatment of the LCR rats did improve glycolytic skeletal muscle insulin sensitivity that was reduced by a 3 d HFD challenge.

The heat shock response (induction of HSPs) following exercise or heat treatment has been shown to be either blunted or enhanced in diabetic rodents (17, 236, 346). The induction of HSP72 following heat treatment was similar in the glycolytic skeletal muscle between HCR/LCR rats, but was absent in the oxidative skeletal muscle of the LCR rats. We also observed a blunted induction of HSPs in the skeletal muscle and liver of LCR rats fed a HFD challenge for 3 d. The blunted induction of HSPs in LCR rats may represent a compromised stress response, and this aberrant stress response may actually play a direct role the differences in endurance capacity between the HCR/LCR rats. Future experiments in the HCR/LCR rats will continue to explore whether the blunted heat shock response is also present following acute and chronic exercise, and to determine whether chronically improving HSP levels improve insulin sensitivity leading to improved oxidative capacity.

Whether the heat shock response is blunted in the WAT during insulin resistance or high-fat feeding has not been tested. The induction of HSPs in the BAT following cold exposure is

blunted in diabetic rats (308). In separate experiments, we have observed a strong induction of HSP72 in the SCAT of rats fed a chow diet, but this induction was not as pronounced in rats fed a HFD for 15 wk. To test whether insulin resistance blunts the WAT heat shock response, the HCR/LCR rats are an excellent model, and we will test whether the induction is blunted in LCR rats and exacerbated by high-fat feeding. In addition, we will test for differences constitutive levels of HSPs between HCR/LCR rats in WAT.

To further examine the potential of HSPs to protect against metabolic insult, we have examined whether weekly *in vivo* heat treatments preceding a 3 d HFD challenge protect against metabolic derangements. In male Wistar rats, 6 wk of heat treatments preceding a 3 d HFD did not alter weight gain (**Figure 23A**) or food intake (data not shown), but significantly reduced glucose intolerance (**Figure 23B**). This experiment will further serve to test whether heat treatments can alter mitochondrial functionality in the liver – where we believe the largest metabolic derangements occur in response to an acute HFD challenge. The role HSPs may play in protecting the liver from metabolic dysfunction and steatosis has not been widely studied.

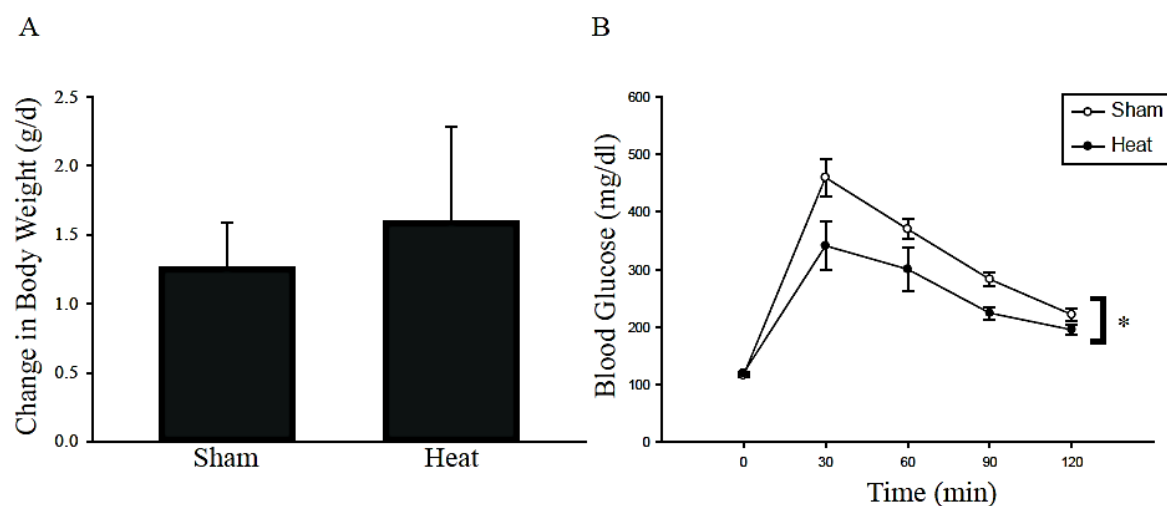


Figure 23. Six wk of weekly *in vivo* heat treatments reduce glucose intolerance following a 3 d HFD challenge. (A) Changes in body weight per day during the 3 d HFD challenge and (B) blood glucose response to an intraperitoneal glucose tolerance test in male Wistar rats receiving 6 wk of either *in vivo* sham (37°C, 20 min) or heat (42°C, 20 min) treatment prior to the initiation of a 3 d HFD challenge. Rats were fasted for 8 hr following 3 nights of high-fat feeding and were injected with a glucose load of 2 g/kg body weight intraperitoneally. Blood glucose was measured prior to, and 30, 60, 90, and 120 min following injecting using a glucometer. * $P < 0.05$ denotes a significant main effect difference between treatment groups determined by a 2-way repeated measures ANOVA. Values are mean \pm SEM. N = 5-6 animals/group.

5.4 ROLE OF HSPS AND HEAT TREATMENT IN PROTECTING THE LIVER FROM STEATOSIS

Very little is known regarding the metabolic role of HSPs in the liver. We observed that in of rats fed a HFD, weekly heat treatments reduced triglyceride accumulation in the liver. While this is a simple observation, it is important to remember that the accumulation of excess lipids in the liver is paramount in the development of NAFLD and hepatic steatosis (49, 326, 401). Multiple pro-inflammatory stress kinases have been shown to be active in the liver of insulin resistant animals (PKC, IKK β , and JNK) (428). Our laboratory has shown that HSP72 induction in skeletal muscle can reduce JNK phosphorylation (154, 155), and other laboratories

have shown that heat treatment or pharmacological induction of HSP72 reduce hepatic triglyceride accumulation, inflammation, and increase insulin responsiveness (3, 330, 331). In the liver of LCR rats fed a 3 d HFD challenge where JNK phosphorylation is increased, we observed that heat treatment reduced JNK phosphorylation. These observations have generated new hypotheses in our laboratory that we are currently pursuing.

As mentioned previously, we are currently investigating whether 6 wk of weekly *in vivo* heat treatments prevent metabolic derangements induced by a 3 d HFD challenge. We have focused this investigation on the liver where we believe that HSP72 will protect against lipid accumulation by modulating mitochondrial functionality and mitophagy directly. The use of HSP72 knockout models could allow us to mechanistically test the role of HSP72 in protecting against lipid accumulation. We hypothesize that lipid accumulation and inflammation will be exacerbated in HSP72 knockout mice fed a HFD. HSP72 could also be overexpressed via transgenic means or adenoviral delivery, and we would hypothesize this would potentially protect mice from HFD induced steatosis and inflammation.

5.5 HSP72'S ROLE IN MITOCHONDRIAL BIOGENESIS, AUTOPHAGY, AND MITOPHAGY

HSP72 overexpression and heat treatment has been shown to increase mitochondrial biogenesis and activity of mitochondrial enzymes in skeletal muscle and muscle cells (59, 67, 173, 293, 471), and these adaptations may also occur in the liver and WAT. HSP72 has been shown to exert influence over autophagy-mitophagy. The ability of Pink-1 to direct Parkin to the mitochondrial membrane to initiate mitochondrial degradation is impaired in the absence of

HSP72 leading to enhanced mitochondrial fusion and accumulation of dysmorphic mitochondria (101).

PGC-1 α has been touted as the master regulator of mitochondrial biogenesis (394). Heat treatment of C2C12 muscle cells increases Sirt1 and PGC-1 α expression ultimately resulting in increased levels of respiratory chain complexes (293). HSP72 overexpression increases mitochondrial biogenesis and increases Sirt1 levels and AMPK activity, both of which are involved in the regulation of PGC-1 α (173, 271). WAT mitochondrial biogenesis is suppressed in high-fat fed and *db/db* mice, but can be increased in WAT by exercise training (414, 468). We observed that PGC-1 α was increased in the WAT following heat treatment in high-fat fed rats. Should the WAT mitochondrial content increase, this would allow for increased fatty acid oxidation to occur in the adipocytes themselves influencing other aspects of metabolism as well. It will be necessary to measure the protein levels of mitochondrial respiratory chain complexes or other mitochondrial enzymes (i.e., citrate synthase) to follow-up on our observations in PGC-1 α expression. As only approximately 10% of fatty acid oxidation occurs in the adipose tissue itself, changes in mitochondrial fatty acid oxidation in the adipose tissue will likely have only modest effect on whole-body metabolism. Interestingly, endurance exercise is a well-established means to increase mitochondrial content and HSP72 levels in skeletal muscle (182, 184, 334, 354) and increases mitochondrial content in adipose tissue (468). However, only a single report has investigated whether exercise increases HSP content in WAT finding that 6 wk of voluntary wheel running did not increase HSP levels in WAT. The induction of HSP in WAT may require greater intensity than voluntary wheel running would provide.

In the skeletal muscle of high-fat fed mice, mitochondrial biogenesis occurs to compensate for excess dietary lipids (161, 495), and our laboratory has similarly observed this (154). In the

skeletal muscle of heat treated high-fat fed rats, we observed reductions in PGC-1 α levels and increased activity of mitochondrial enzymes (i.e., citrate synthase and cytochrome oxidase) without changes in the content of respiratory chain complexes (unpublished observations). This may illustrate that increased HSP72 levels increase the degradation of damaged mitochondria via autophagy-mitophagy leading to a reduced requirement for mitochondrial biogenesis.

Supporting a role for HSP72 in regulating mitophagy, we also observed potentially dysfunctional mitophagy in the HCR/LCR rats. In LCR rats where HSP72 levels are low, Parkin levels are increase potentially to compensate for dysfunctional mitophagy, similar to what has been observed in HSP72 heterozygous and homozygous knockout mice (101). This observation may also represent a new mechanism by which the HCR rats display greater mitochondrial content and functionality compared to LCR rats. As opposed to just simply higher levels of mitochondrial biogenesis (as we did observe higher PGC-1 α expression in HCR rats), the LCR rats may be less able to clear damaged mitochondria due to lower HSP72 levels and dysfunctional Parkin expression.

HSP72 appears to have a direct role in modulating autophagy itself and may protect against metabolic derangements. More specifically, autophagy has been shown to be essential for mobilizing fatty acids from the lipid droplets of hepatocytes – a process termed lipophagy (397, 451). We observed that markers of autophagy are greater in the liver of heat treated rats compared to sham treated rats fed a HFD. This observation may represent that autophagy is more active following heat treatment liberating fatty acids for oxidation in the mitochondria of the liver. We also observed that in HCR rats, where HSP72 levels are higher, autophagy (LC3-II levels) was increased in response to a 3 d HFD, which did not occur in LCR rats where HSP72 levels are lower. This would be the first report to show that a 3 d HFD challenge increases

autophagy in the skeletal muscle, and we believe that this is to redistribute fatty acids from skeletal muscle lipid droplets to the mitochondria to be oxidized. We plan to investigate further the autophagy machinery in the HCR/LCR rats to attempt to determine where the benefit or defect between the strains has occurred.

5.6 ORGAN CROSSTALK IS IMPROVED BY CHANGING HSP LEVELS

The crosstalk between organs has become of great interest. HSP72 is released into the circulation following exercise from the liver, but not the muscle (111, 114, 506), but its role in communication between organs has not been well described. HSP60 has been shown to be released from the adipose tissue and play a pro-inflammatory role in adipose tissue and skeletal muscle (150, 302, 303). The influence of HSPs between organs may not be due to circulating levels of the HSPs themselves, but in their ability to improve homeostasis of organs under stress. For instance, transgenic overexpression of HSP72 in skeletal muscle reduced epididymal fat pad mass, increased WAT lipolysis, increased circulating adiponectin concentration, and reportedly (although not published) reduced hepatic triglyceride and DAG content (67, 173, 175). Induction of HSP72 using GGA resulted in reduced leptin concentration in high-fat fed mice (3). Heat treatment may also induce changes in the hormonal milieu that lead to changes in insulin sensitivity. Heat treatment of isolated adipocytes has been shown to positively influence gene expression and secretion of adiponectin and leptin in a way that should have a positive influence if these effects also occurred *in vivo* (33). How heat treatment itself, but also the HSP72 themselves, alter the communication networks between organs is another interesting avenue for our work.

5.7 MODULATORS OF HSPS AS TREATMENT MODALITIES

Exercise is a potent inducer of HSPs in the skeletal muscle and liver, and exercise is a first line strategy for treating and preventing insulin resistance and type 2 diabetes.

Unfortunately, the prevalence of physical inactivity has grown and not enough individuals meet the guidelines for daily exercise and physical activity. Due to the insulin sensitizing effects of heat treatment, a number of molecules that induce HSPs are being explored as treatment options for diabetes (reviewed by (190)). However, the widespread use of *in vivo* heat treatments in humans is problematic as many insulin resistant individuals are also in poor health and may be negatively impacted by heat stress. As mentioned previously, GGA (a drug used for treating gastric ulcers) induces HSP72 and decreases inflammation, adipose tissue mass, and increases insulin sensitivity (3, 235). Our own laboratory has shown that lipoic acid, a HSP co-inducer that has anti-inflammatory effects leading to improved glucose tolerance and skeletal muscle sensitivity in high-fat fed rats (153). Drugs that inhibit HSP90, resulting in HSF-1 activation and induction of HSPs, have also been used to treat diabetic rodents (109). BGP-15, a hydroximic acid that enhances membrane fluidity, has reached clinical trial status in Australia, and has been used to reduce glucose intolerance, inflammation, and increase mitochondrial mass in rodents; but has also been used to treat other diseases such as muscular dystrophy (67, 134, 257, 292). The use of compounds that activate the heat shock response is exciting and a new avenue for the treatment of insulin resistance.

5.8 CONCLUSIONS

The induction of HSPs has been shown to improve glucose intolerance and skeletal muscle insulin sensitivity. In this work, we further confirm these observations and begin to establish a protective role of HSPs in preventing hepatic steatosis, possibly by modulating the autophagic process. We establish that HSF-1, HSP72, and HSP60 are lower in an animal model of insulin resistance concurrent with low intrinsic aerobic capacity, and lower constitutive HSP72 and HSP60 levels or a blunted heat shock response may play a direct role in oxidative capacity. The induction of the HSP response should not be ignored as a potential treatment option for insulin resistant individuals with low CRF.

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